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(54) Title: DNA ENCODING A HUMAN SUBUNIT OF THE 5-HT3 SEROTONIN RECEPTOR

(57) Abstract: DNA encoding human 5-HT3-B has been cloned and characterized. The recombinant protein is capable of forming biologically active human 5-HT3-B protein. The cDNA has been expressed in recombinant host cells that produce active recombinant protein. In addition, the recombinant host cells are utilized to establish a method for identifying modulators of the receptor activity, and receptor modulators are identified.

TITLE OF THE INVENTION

DNA ENCODING A HUMAN SUBUNIT OF THE 5-HT3 SEROTONIN RECEPTOR

BACKGROUND OF THE INVENTION

Serotonin (5-hydroxytryptamine, 5-HT) is a multifunctional chemical transmitter that signals though cell surface receptors. At least fourteen subtypes of serotonin receptors have been defined pharmacologically (Julius, 1996; Tecott and Julius, 1993). Thirteen of the fourteen known receptors are G-protein coupled receptors and the only known ionotropic 5-HT receptor, the type 3 5-HT3 receptor, is a fast activating, ligand gated non-selective cation channel unique among known monoamine receptors (Derkach et al., 1989). The 5-HT3 receptor is exclusively localized on neurons in the central (Waeber et al., 1989; Yakel et al., 1991) and peripheral (Fozard, 1984) nervous systems. Activation of the 5-HT3 receptor leads to membrane depolarization and an increase in intracellular Ca^{2+}. The 5-HT3 receptor is the target of antagonists (granisetron and ondansetron) selective against the nausea induced by cytotoxic chemotherapy and general anesthesia (Gralla, 1998). Evidence is accumulating that serotonin 5-HT3 receptors are important in pain reception, anxiety, cognition, cranial motor neuron activity, sensory processing, modulation of affect, and the behavioral consequences of drug abuse (Lambert et al., 1995; Passani and Corradetti, 1996).

The 5-HT3 receptor is thought to be a homopentimeric protein with multiple agonist and allosteric ligand binding sites (Boess et al., 1995; Bonhaus et al., 1995; Eglen and Bonhaus, 1996; Green et al., 1995; Hargreaves et al., 1996; Lambert et al., 1995; Van Hooft et al., 1997; Wetzel et al., 1998). The full coding sequence of the 5-HT3 receptor has been cloned from mouse (Hope et al., 1993; Maricq et al., 1991; Werner et al., 1994), rat (Miyake et al., 1995), guinea pig (Lankiewicz et al., 1998), and human (Belelli et al., 1995; Miyake et al., 1995); AJ003079 Bruss et al., unpublished). It has structural and functional similarities with nicotinic, GABA-ergic and other ligand gated ion channels (Barnard, 1996; Gurley and Lanthorn, 1998;

Maricq et al., 1991). Like other receptors of the same ligand-gated cation channel superfamily (Changeux and Edelstein, 1998; Lena and Changeux, 1993), the 5-HT3 receptors rapidly desensitize (Peters and Lambert, 1989; Yakel et al., 1991). The pharmacological and kinetic profile of the members of this superfamily depends on subunit composition (Chang et al., 1995; Harris et al., 1995; Lindstrom et al., 1990; Luetje and Patrick, 1991; Olsen, 1998).

While it is thought that only one gene encoding the 5-HT3 receptor exists (5-HT3-A receptor), several lines of evidence indicate that 5-HT3 receptors may exist as heteromultimers. First, receptors purified from a variety of sources by affinity chromatography usually reveal at least 2 major protein bands with molecular masses in the order of 54 and 38 kDa (Lambert et al., 1995). The 5-HT3-A receptor corresponds to the former (Turton et al., 1993). Affinity purified 5-HT3 receptor solubilized from pig cerebral cortex is composed of at least 3 separable components, based on silver staining of proteins on denaturing gels (Fletcher and Barnes, 1997). A number of these protein bands are not recognized by specific antibodies directed against the recombinant 5-HT3-A subunit (Fletcher and Barnes, 1997), and their sizes are too large (52-71 kDa) to be considered as degraded 5-HT3-A fragments (Fletcher and Barnes, 1998).

Second, expression of the recombinant receptor in Xenopus oocytes or mammalian cell lines often do not reveal all the electrophysiological and pharmacological properties of the native receptor (Gill et al., 1995; Lambert et al., 1995; Van Hooft et al., 1997). Differences in desensitization kinetics, single channel conductance and agonist efficacy have been observed and may be due to the lack of an endogenous subunit not present in the recombinant cell lines or oocyte system. Two forms of the receptor subunit with about 98% identity have been observed in mouse, rat, guinea pig and human. The two forms differ by the insertion of 6 to 32 consecutive amino acids and may be produced by alternative splicing of a single gene (Uetz et al., 1994; Werner et al., 1994). While most of the pharmacological and electrophysiological characteristics of the recombinant mouse isoforms are similar (Downie et al., 1994; Niemeyer and Lummis, 1998; Werner et al., 1994), the efficacy

of 2-methyl-5-HT and/or m-chlorophenylbiguanide (mCPBG) differ (Downie et al., 1994; Niemeyer and Lummis, 1998; Van Hooft et al., 1997). The two variants may coexist in the same cell lines and heteromultimers containing both forms may explain some but not all of the differences observed between homomultimers of recombinant subunits and the native cells.

Third, 5-HT-3 receptors in different preparations from the same species reveal variation in electrophysiological and pharmacological properties (Fletcher and Barnes, 1998; Richardson and Engel, 1986). In particular, there appears to be heterogeneity of desensitization kinetics across cells, and even within the same cell type (Lambert et al., 1995). Differences in desensitization kinetics have been observed in NG108-15 cells under various differentiation states (Shao et al., 1991). While this difference may be due to heterogeneity of subunits, it may also be a consequence of different post-translational states of the receptor. For instance, the rate of desensitization of nicotinic acetylcholine and $GABA_A$ receptors is enhanced by phosphorylation (Raymond, 1998; Raymond et al., 1993; Swope et al., 1992). A wide range of single channel conductance values has been reported for the 5-HT3 receptor (Fletcher and Barnes, 1998), however, this difference may be due to the phosphorylation state of the receptor in different cells. Van Hooft and colleagues have shown that phosphorylation controls the conductance of 5-HT-3 receptors in N1E-115 cells (Van Hooft and Vijverberg, 1995). Furthermore, significant differences in rectification properties of the channel have been reported in different cell types (Hussy et al, 1994).

Fourth, differentiated murine N1E-115 cells express native 5-HT-3 receptors with an efficacy for 2-methyl-5-HT higher than that observed for either murine recombinant 5-HT3-A isoform expressed in oocytes separately or together. However, co-expression of recombinant 5-HT3-A receptor in oocytes with mRNA isolated from differentiated murine N1E-115 cells reconstitutes the functional properties of the native receptor expressed on differentiated cells (Van Hooft et al., 1997).

While it has been reported that the 5-HT3 receptor can co-assemble with the nicotinic alpha4 subunit in Xenopus oocyte expression studies (Van Hooft et al.,

1998), nicotinic ACh receptor subunits were not associated with native porcine brain 5-HT3 receptors (Fletcher et al., 1998).

Fifth, allosteric modulation of the receptor by Zn^{2+} has different effects on the recombinant murine 5-HT3-A receptor expressed in oocytes (enhancement) compared to its effects on 5-HT receptor-mediated currents in NCB20 cells (block; (Lovinger, 1991), the cell line from which the 5-HT3-A receptor used in these studies was cloned. The sensitivity of another member of this superfamily of ligand-gated receptors, $GABA_A$ receptors, to blockade by zinc ions is known to depend on subunit composition (Smart et al., 1994).

Recently the sequence of human 5-HT3-B was disclosed and shown to alter functional characteristics of 5-HT3-A (Davies et al 1999). The functional characteristics described for human 5-HT3-B by Davies et al. (1999) included a decreased affinity for 5-HT with no effect on the affinity for other agonists including mCPBG. This art did not find that the affinity and cooperativity for 5-HT3 receptor function were increased and decreased, respectively, by 5-HT3-B, nor did it report any affects on current kinetics.

The isolation and functional characterization of a human cDNA encoding a modifier subunit for the serotonin 5-HT3 receptor explains observations that 5-HT3 receptors from a variety of preparations have distinct pharmacological, kinetic and voltage-dependent properties (Peters et al., 1992). Expression of this human 5-HT3 subunit, termed 5-HT3-B will further aid in discovery of serotonin function and can be used to screen for compounds that modulate function of a heteromeric receptor complex to which the 5-HT3-B receptor participates.

SUMMARY OF THE INVENTION

A DNA molecule encoding a human subunit with homology to 5-HT3-A serotonin receptor that, when co-expressed with the short form of the serotonin 5-HT3-A receptor, modifies the functional and pharmacological characteristics of the 5-HT3 receptor has been cloned and characterized. Using a recombinant expression system, functional DNA molecules encoding the human serotonin 5-

HT3 receptor modifier protein (heretofore designated 5-HT3-B) have been isolated. The biological and structural properties of these proteins are disclosed, as is the amino acid and nucleotide sequence. The recombinant protein is useful to identify modulators of human 5-HT3 receptors composed of both 5-HT3 receptors and the modifier subunit (5-HT3-B). Modulators identified in the assay disclosed herein are useful as therapeutic agents, include, but are not limited to, nausea, depression, anxiety, psychoses (for example schizophrenia), urinary continence, Huntington's chorea, tardive dyskinesia, Parkinson's disease, obesity, hypertension, migraine, Gilles de la Tourette's syndrome, sexual dysfunction, drug addiction, drug abuse, cognitive disorders, learning, Alzheimer's disease, cerebral coma, senile dementia, obsessive-compulsive behavior, panic attacks, pain, social phobias, eating disorders and anorexia, cardiovascular and cerebrovascular disorders, non-insulin dependent diabetes mellitus, hyperglycemia, constipation, arrhythmia, disorders of the neuroendocrine system, stress, and spasticity, as well as acid secretin, ulcers, airway constriction, asthma, allergy, inflammation, and prostate dysfunction, and diagnostic agents. The recombinant DNA molecules and portions thereof, are useful for isolating homologues of the DNA molecules, identifying and isolating genomic equivalents of the DNA molecules, and identifying, detecting or isolating mutant forms of the DNA molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 – [SEQ.ID.NO.:5] Nucleic Acid sequence of the human 5-HT3-B (full sequence including untranslated regions) is shown;1923 bases (141 bases of 5' UTR; 456 bases of 3' UTR).

Figure 2 - [SEQ.ID.NO.:6] Nucleotide sequence for the coding region of 5-HT3-B is shown; 1326 bases.

Figure 3 - [SEQ.ID.NO.:7] The amino acid sequence of human 5-HT3-B is shown (441 amino acids).

Figure 4 – PANEL A [Figure 4A]. The PCR-based tissue distribution of the human 5-HT3-B is shown. Lanes are labeled as indicated: 1, Cerebellum; 2, Cerebral cortex; 3, Medulla; 4, Spinal Cord; 5, Occipital pole; 6, frontal lobe; 7, Temporal lobe; 8, Putamen; 9, Amygdala; 10, Caudate nucleus; 11, Corpus callosum; 12, Hippocampus; 13, Whole brain; 14, Substantia nigra; 15, Thalamus; 16, Heart; 17, Brain; 18, Placenta; 19, Lung; 20, Liver; 21, Skeletal muscle; 22, Kidney; 23, Pancreas; 24, Spleen; 25, Thymus; 26, Prostate; 27, Testis; 28, Ovary; 29, Small Intestine; 30, Colon (mucosal lining); 31, Peripheral blood leukocyte.

PANEL B [Figure 4B]. RT-PCR in situ hybridization was performed on normal human tissues of the spleen (b1, b2) and small intestine (b3, b4). Human spleen had intense 5-HT3-B mRNA (b1) and 5HT3-A mRNA (b2) signal (purple in color – dark grey in black-and-white), respectively, in small lymphocytes (arrows). Similarly, intense 5-HT3-B mRNA (b3) and 5HT3-A mRNA (b4) signal (purple in color – dark grey in black-and-white) was detected in stromal fibroblasts of the small intestines (arrows). Total magnification = 600x.

PANEL C [Figure 4C]. RT-PCR in situ hybridization was performed on serial 10 μm sections of the normal monkey amygdala. Figures 4c1 (300x) and 4c3 (600x) show positive 5-HT3-A mRNA signal (purple in color – dark grey in black-and-white) in seven neurons (arrows) which is also show positive 5HT3-B mRNA signal (4c2 (300x); 4c4 (600x)). Not all cells were labeled by the RT-PCR ISH technique using specific probes for 5-HT3-B or 5-HT3-A.

Figure 5 - PANEL A [Figures 5A and 5B]. Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: 5-HT3-B modifies the kinetics and magnitude of 5-HT3-A currents elicited by a subset of agonists. (a.) 5-HT3-B normalizes agonist-induced 5-HT3-A responses in *Xenopus* oocytes. 5-HT3-A-expressing oocytes responded to 5-HT, 2-methyl-5-HT, mCPBG and 1-PBG with either a slowly desensitizing response (left panel; "slow" responders, 71% of oocyte batches), or a complex current that included a rapidly desensitizing

component (right panel; "fast" responders). Oocytes expressing both subunits produced uniform responses (middle panel). The agonists tested included 5-HT (0.3 and 10 μM, responses are superimposed); mCPBG (0.3 and 10 μM); 1-PBG (10 and 100 μM); DA (0.1 and 1 mM); 2-Me-5-HT (10 μM). Agonists are applied during the time indicated by the horizontal bar above the record. The clear bar extending from the solid bar indicates the longer exposure to agonist for one of the superimposed traces. Oocytes were continuously perfused with Ba^{2+} containing saline at a rate of 10 ml/min at room temperature. Time scale bar: 40 sec. (b.) Responses to 1-PBG were blocked by the 5-HT3 antagonist tropisetron. After obtaining a control response to 1-PGB (100 μM; indicated by the solid bar) (left panel), the oocyte was washed 2 min then incubated for 30 sec in tropisetron (1 μM; clear bar) then challenged with both 1-PBG and tropisetron. The response to 1-PBG was completely blocked (middle panel). The response to agonist recovered after a 2 min washout of antagonist (right panel).

PANEL B [Figure 5C]. Peak currents elicited by the indicated agonists from oocytes expressing 5-HT3-A (grey bars) and both 5-HT3-B and 5-HT3-A (solid bars). Data from oocytes recorded in Ba^{2+}- and Ca^{2+}- containing saline were combined since there were no differences in 5-HT (10 μM)-induced maximal current in Ca^{2+}-containing vs. Ba^{2+}-containing saline (-7.7 +/- 1.4 μA (n=16) vs. -7.6 +/- 0.9 μA (n=24)). The number of oocytes tested ranged from 9 to 61; SEM values were 10% for 5-HT (10 μM) and 21% for both mCPBG (10 μM) and 1-PBG (100 μM). p values are indicated: * $p<0.05$, ** $p<0.01$ (Students t-test).

Figure 6 - Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: 5-HT3-B normalized the 5-HT response t80 and time to peak to similar values in "slow" (a,c) and "fast" (b,d) responders. (a.) t80 of responses elicited by 5-HT (concentrations indicated) from experiments in which 5-HT3-A-injected oocytes respond to 5-HT with a slowly decaying

response. 5-HT3-A-injected oocytes (grey bars; n were 8, 38 and 13 individual oocytes, respectively); 5-HT3-A receptor and 5-HT3-B -injected oocytes (solid bars; n were 11, 33 and 16 individual oocytes, respectively). The differences were significant at all agonist concentrations ($p< 0.05$, $5e^{-9}$, 0.005 for 1, 10 and 100 μM 5-HT, respectively). (b.) t80 of responses elicited by 5-HT from experiments in which 5-HT3-A-injected oocytes respond to 5-HT with a rapidly decaying response. 5-HT3-A injected oocytes (grey bars; n were 5, 11 and 10 individual oocytes, respectively); 5-HT3-A receptor and 5-HT3-B injected oocytes (solid bars; n were 9, 12 and 3 individual oocytes, respectively). Significant differences were observed at 1 and 10 μM 5-HT ($p < 0.005$ and $p< 5e^{-5}$, respectively). (c.) The time to peak of the response to 10 μM 5-HT in slow responders is significantly faster when 5-HT3-B is co-expressed with 5-HT3-A (solid bars, n= 26) compared to 5-HT3-A alone (grey bars, n=41, $p<0.0005$). (d.) The time to peak of the response to 10 μM 5-HT in "fast" responders was significantly slower when 5-HT3-B is co-expressed (solid bars, n=12) compared to homomers (grey bars, n=10, $p<0.05$).

Figure 7 - Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: Agonist dose response relationships are altered in the presence of 5-HT3-B and depend on the ratio of 5-HT3-B to 5HT3-A cRNA injected in Xenopus oocytes. (a.) Oocytes were injected with either 5-HT3-A or both 5-HT3-A and 5-HT3-B cRNA and tested for their response to the indicated concentrations of 5-HT. The data are presented relative to the maximum response elicited by 100 μM 5-HT in the same oocytes. 5-HT3-B decreases the apparent affinity of the 5HT3-A receptor for 5-HT. The agonist was applied to the cell at a rate of 10 ml per min in bath perfusate. The data were analyzed using GraphPad Prizm and fit with a Boltzmann exponential. (b.) The percent maximum response for 0.3 μM 5-HT, 0.3 μM mCPBG and 10 μM 1-PBG are plotted for oocytes injected with 5-HT3-A alone (left-most stippled bars), both subunits at a 1:10 ratio (5-HT3-A to 5-HT3-B, solid bars), both subunits at a 1:1 ratio (hatched bars), both subunits at a 1:0.1 ratio (clear

bars), and both subunitis at a 1: 0.01 ratio (cross-hatched bars). The percent of the maximal response obtained in individual oocytes was averaged. Significant differences are indicated by asterisks (* $p<0.05$; ** $p< 0.01$).

Figure 8- Specificity of the modulatory effect of human 5-HT3-B in oocytes is shown. In all cases the amount of injected 5-HT3-B cRNA was at least 2-fold the concentration of each nACh receptor. (a.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha3\beta4$ with (solid; n= 5) and without (grey; n=4) 5-HT3-B. (b.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha4\beta2$ with (solid; n=8) and without (grey; n=9) 5-HT3-B. (c.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha2\beta2$ with (solid; n=3) and without (grey; n=3) 5-HT3-B. (d.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha7$ with (solid; n=12) and without (grey; n=15) 5-HT3-B.

Figure 9 - PANEL A [Figures 9A and 9B]. Functional expression of human 5-HT3-B in recombinant host cells is shown: 5-HT3-B and 5-HT3-A heteromers display pharmacological and voltage-dependent properties distinct from 5-HT3-A homomeric receptors. (a.) Ca^{2+} influx induced by agonist challenge was determined using the Ca^{2+} sensitive dye Fluo-4 on the FLIPR system. Responses to 0.17 and 3 μM 5-HT (top), 6 and 30 μM 1-PBG (middle), 0.3 and 3 μM mCPBG are shown. Each record represents 3.33 min. Agonists were added after 20 seconds and were present throughout the recording. Left: 5-HT3-A/HEK cells; Right: 5-HT3-A/5-HT3-B/HEK cells. (b.) Dose response for 5-HT - activated Ca^{2+} influx using the FLIPR system. 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) had significantly higher affinity toward 5-HT and decreased nH compared to 5-HT3-A homomeric receptors (squares). Peak responses were determined and normalized to the maximum observed response.

Mean values from 4-10 separate experiments are presented. Data were fit with Boltzmann exponentials.

PANEL B [Figure 9C]. Dose response for mCPBG-activated Ca^{2+} influx using the FLIPR system. Data from 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) and 5-HT3-A homomeric receptors (squares) is shown. Peak responses were determined and normalized to the maximum observed response. Values were averaged from 4 separate experiments. Data were fit with Boltzmann exponentials.

PANEL C [Figures 9D and 9E]. Voltage clamp recordings from 5-HT3-A/HEK cells (left) and 5-HT3-A/5-HT3-B/HEK cells (right). Spikes in the current record are the currents induced by voltage ramp protocols used to determine the change in whole cell membrane conductance. Voltage ramps were evoked every second. (d.) Top: inward currents elicited by 10 μM 1-PBG (solid bar) and 10 μM 5-HT (clear bar) from a holding potential of -68 mV. Bottom: inward currents elicited by 100 μM 1-PBG (hatched bar). (e.) The voltage relationship for the agonist induced currents obtained using a voltage ramp protocol. 5-HT induced currents from transfected HEK293 cells expressing the homomeric receptor (left) and 5-HT3-A and 5-HT3-B subunits (right). A voltage ramp was applied to the cell from -120 to +80 mV (not corrected for a junction potential of -18 mV) over 200 msec (1 mV/ms) before (solid circle) and during (clear circle) agonist application. The V_{rev} were similar for both responses and were near 0 mV. Asterisks above the recordings in (d) indicate the ramp currents shown on an expanded scale in (e).

DETAILED DESCRIPTION

The present invention relates to DNA encoding human 5-HT3-B, which was isolated from a cDNA library from human small intestine. The human 5-HT3-B, as used herein, refers to protein, which can specifically function as a receptor in a complex with the 5-HT3-A receptor.

The complete amino acid sequence of the human 5-HT3-B was not known, nor was the complete nucleotide sequence encoding human 5-HT3-B known prior to the cloning and functional determination of the present invention. However, the cloning of a full length DNA molecule encoding human 5-HT3-B and some aspects of the function of the protein encoded by this molecule was recently reported (Davies et al., 1999).

The present invention provides a human 5-HT serotonin receptor complex that has distinct pharmacological, kinetic and voltage-dependent properties, which mimic native responses to a greater degree than previously described. Thus the invention described herein shows that 5-HT3-B confers distinct pharmacological, kinetic and voltage-dependent properties upon the 5-HT3-A receptor. The present invention shows that 5-HT3-B specifically interacts with the 5-HT3-A and not the nicotinic ACh receptors $\alpha 3\beta 4$, $\alpha 2\beta 2$, $\alpha 4\beta 2$ and $\alpha 7$. Furthermore, the mRNA encoding the invention described herein is co-localized within neurons in the monkey amygdala and human cerebral cortex. The present invention further shows that 5-HT3-B and 5-HT3-A mRNA are expressed in lymphocytes and epithelial cells of peripheral organs including the spleen and small intestine. The physiological significance of the novel findings reported herein include the ability of cells co-expressing 5-HT3-B and 5-HT3-A to be more sensitive to 5-HT than cells expressing single receptors, and to have a altered response duration to agonist. These alterations in receptor-mediated current could have profound effects on 5-HT activation of neuronal excitability. It is predicted that a wide variety of cells and cell types will contain the human 5-HT3-B. Vertebrate cells capable of producing human 5-HT3-B include, but are not limited to human 5-HT3-B -expressing cells isolated from cells that show sensitivity to or bind serotonin.

Other cells and cell lines may also be suitable for use to isolate human 5-HT3-B cDNA. Selection of suitable cells may be done by screening for the response to 1-phenylbiguanide (1-PBG) or mCPBG, either the magnitude of the response at low micromolar concentrations of 1-PBG or mCPBG, or the rate of

decay of the cellular response elicited by 1-PBG, mCPBG, or serotonin. Human 5-HT3-B activity can be monitored by performing a ^{3}H- [mCPBG] binding assay in the presence of 5-HT3-A receptor (Steward et al., 1993), by direct measurement of a Ca^{+2} influx using the Ca^{+2} sensitive dyes (Kuntzweiler et al., 1998), or by net ion flux using voltage clamp techniques (Hamill et al., 1981). Cells that possess isolate human 5-HT3-B activity in this assay may be suitable for the isolation of isolate human 5-HT3-B DNA or mRNA.

Any of a variety of procedures known in the art may be used to molecularly clone human 5-HT3-B DNA. These methods include, but are not limited to, direct functional expression of the human 5-HT3-B genes following the construction of a human 5-HT3-B-containing cDNA library in an appropriate expression vector system. Another method is to screen human 5-HT3-B -containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a labelled oligonucleotide probe designed from the amino acid sequence of the human 5-HT3-B subunits. An additional method consists of screening a human 5-HT3-B -containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA encoding the human 5-HT3-B protein. This partial cDNA is obtained by the specific PCR amplification of human 5-HT3-B DNA fragments through the design of degenerate oligonucleotide primers from the amino acid sequence of the purified human 5-HT3-B protein.

Another method is to isolate RNA from human 5-HT3-B -producing cells and translate the RNA into protein via an *in vitro* or an *in vivo* translation system. Translation of the RNA into a peptide a protein will result in the production of at least a portion of the human 5-HT3-B protein which an be identified by, for example, immunological reactivity with an anti- human 5-HT3-B antibody or by biological activity of human 5-HT3-B protein. In this method, pools of RNA isolated from human 5-HT3-B -producing cells can be analyzed for the presence of an RNA that encodes at least a portion of the human 5-HT3-B protein. Further fractionation of the RNA pool can be done to purify the human 5-HT3-B RNA from non- human 5-HT3-B RNA. The peptide or protein produced by this

method may be analyzed to provide amino acid sequences which in turn are used to provide primers for production of human 5-HT3-B cDNA, or the RNA used for translation can be analyzed to provide nucleotide sequences encoding human 5-HT3-B and produce probes for this production of human 5-HT3-B cDNA. This method is known in the art and can be found in, for example, Maniatis, T., Fritsch, E.F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989.

It is readily apparent to those skilled in the art that other types of libraries, as well as libraries constructed from other cells or cell types, may be useful for isolating human 5-HT3-B -encoding DNA. Other types of libraries include, but are not limited to, cDNA libraries derived from other cells, from organisms other than human, and genomic DNA libraries that include YAC (yeast artificial chromosome) and cosmid libraries.

It is readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from cells or cell lines which have human 5-HT3-B activity. The selection of cells or cell lines for use in preparing a cDNA library to isolate human 5-HT3-B cDNA may be done by first measuring cell-associated human 5-HT3-B activity using the measurement of human 5-HT3-B -associated biological activity or a human 5-HT3-B - 5-HT3-A receptor ligand binding assay [^{3}H-mCPBG] (Steward et al., 1993).

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Maniatis, T., Fritsch, E.F., Sambrook, J., Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

It is also readily apparent to those skilled in the art that DNA encoding human 5-HT3-B may also be isolated from a suitable genomic DNA library. Construction of genomic DNA libraries can be performed by standard techniques well known in the art. Well known genomic DNA library construction techniques can be found in Maniatis, T., Fritsch, E.F., Sambrook, J. in Molecular Cloning: A

Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

In order to clone the human 5-HT3-B gene by the above methods, the amino acid sequence of human 5-HT3-B may be necessary. To accomplish this, human 5-HT3-B protein may be purified and partial amino acid sequence determined by automated sequencers. It is not necessary to determine the entire amino acid sequence, but the linear sequence of two regions of 6 to 8 amino acids from the protein is determined for the production of primers for PCR amplification of a partial human 5-HT3-B DNA fragment.

Once suitable amino acid sequences have been identified, the DNA sequences capable of encoding them are synthesized. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human 5-HT3-B sequence but will be capable of hybridizing to human 5-HT3-B DNA even in the presence of DNA oligonucleotides with mismatches. The mismatched DNA oligonucleotides may still sufficiently hybridize to the human 5-HT3-B DNA to permit identification and isolation of human 5-HT3-B encoding DNA. DNA isolated by these methods can be used to screen DNA libraries from a variety of cell types, from invertebrate and vertebrate sources, and to isolate homologous genes.

Purified biologically active human 5-HT3-B may have several different physical forms. Human 5-HT3-B may exist as a full-length nascent or unprocessed polypeptide, or as partially processed polypeptides or combinations of processed polypeptides. The full-length nascent human 5-HT3-B polypeptide may be posttranslationally modified by specific proteolytic cleavage events that result in the formation of fragments of the full-length nascent polypeptide. A fragment, or physical association of fragments may have the full biological activity associated with human 5-HT3-B however, the degree of human 5-HT3-B

activity may vary between individual human 5-HT3-B fragments and physically associated human 5-HT3-B polypeptide fragments.

The cloned human 5-HT3-B DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant human 5-HT3-B protein. Techniques for such manipulations are fully described in Maniatis, T, et al., supra, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria including E. coli, blue-green algae, plant cells, insect cells, fungal cells including yeast cells, and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungal cells or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one that causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant human 5-HT3-B in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant human 5-HT3-B expression, include but are not limited to, pMAMneo (Clontech), pcDNA3 (InVitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1 (8-2) (ATCC 37110), pdBPV-MMTneo

(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lZD35 (ATCC 37565).

A variety of bacterial expression vectors may be used to express recombinant human 5-HT3-B in bacterial cells. Commercially available bacterial expression vectors that may be suitable for recombinant human 5-HT3-B expression include, but are not limited to pET vectors (Novagen) and pQE vectors (Qiagen).

A variety of fungal cell expression vectors may be used to express recombinant human 5-HT3-B in fungal cells such as yeast. Commercially available fungal cell expression vectors which may be suitable for recombinant human 5-HT3-B expression include but are not limited to pYES2 (InVitrogen) and Pichia expression vector (InVitrogen).

A variety of insect cell expression vectors may be used to express recombinant human 5-HT3-B in insect cells. Commercially available insect cell expression vectors that may be suitable for recombinant expression of human 5-HT3-B include but are not limited to pBlueBacII (InVitrogen).

DNA encoding human 5-HT3-B may be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as E. coli, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to drosophila and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), L-cells, and HEK-293 (ATCC CRL1573).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection,

protoplast fusion, lipofection, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce human 5-HT3-B protein. Identification of human 5-HT3-B expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti- human 5-HT3-B antibodies, and the presence of host cell-associated human 5-HT3-B activity.

Expression of human 5-HT3-B DNA may also be performed using *in vitro* produced synthetic mRNA. Synthetic mRNA or mRNA isolated from human 5-HT3-B producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being generally preferred.

To determine the human 5-HT3-B DNA sequence(s) that yields optimal levels of human 5-HT3-B activity and/or human 5-HT3-B protein, human 5-HT3-B DNA molecules including, but not limited to, the following can be constructed: the full-length open reading frame of the human 5-HT3-B cDNA encoding the 47.9 kDa protein from approximately base 142 to approximately base 1465 (these numbers correspond to first nucleotide of first methionine and last nucleotide before the first stop codon) and several constructs containing portions of the cDNA encoding human 5-HT3-B protein. All constructs can be designed to contain none, all or portions of the 5' or the 3' untranslated region of human 5-HT3-B cDNA. Human 5-HT3-B activity and levels of protein expression may be determined following the introduction, both in combination with 5-HT3-A or alone, of these constructs into appropriate host cells. Following determination of the human 5-HT3-B DNA cassette yielding optimal expression in transient assays, this human 5-HT3-B DNA construct is transferred to a variety of expression vectors, for expression in host cells including, but not limited to, mammalian cells, baculovirus-infected insect cells, E. coli, and the yeast S. cerevisiae.

Host cell transfectants and microinjected oocytes may be used to assay both the levels of human 5-HT3-B activity and levels of human 5-HT3-B protein by the following methods. In the case of recombinant host cells, this involves the co-transfection of one or possibly two or more plasmids, containing the human 5-HT3-B DNA encoding one or more fragments or subunits and the 5-HT3-A receptor or transfection of the human 5-HT3-B protein into human cell lines expressing the 5-HT3-A receptor. In the case of oocytes, this involves the co-injection of synthetic RNAs for human 5-HT3-B and 5-HT3-A receptor proteins. Following an appropriate period of time to allow for expression, cellular protein is metabolically labelled with, for example ^{35}S-methionine for 24 hours, after which cell lysates and cell culture supernatants are harvested and subjected to immunoprecipitation with polyclonal antibodies directed against the human 5-HT3-B protein.

Other methods for detecting human 5-HT3-B activity involve the direct measurement of human 5-HT3-B activity in whole cells transfected with human 5-HT3-A receptor with or without 5-HT3-B cDNA or oocytes injected with human 5-HT3-A receptor and 5-HT3-B mRNA. Human 5-HT3-B activity is measured by specific ligand binding and biological characteristics of the host cells expressing human 5-HT3-B DNA. In the case of recombinant host cells expressing human 5-HT3-A receptor and human 5-HT3-B, patch voltage clamp techniques can be used to measure receptor activity and quantitate human 5-HT3-B protein. In the case of oocytes patch clamp as well as two-electrode voltage clamp techniques can be used to measure the decay rate of agonist-induced currents or agonist dose response.

Levels of human 5-HT3-B protein in host cells are quantitated by immunoaffinity. Cells expressing h5-HT5-B can be assayed for the number of cell surface receptor molecules expressed by measuring the amount of radioactive mCPBG binding to cell membranes. Human 5-HT3-B -specific affinity beads or human 5-HT3-B -specific antibodies are used to isolate for example ^{35}S-methionine labelled or unlabelled human 5-HT3-B protein. Labelled human 5-

HT3-B protein is analyzed by SDS-PAGE. Unlabelled human 5-HT3-B protein is detected by Western blotting, ELISA or RIA assays employing human 5-HT3-B specific antibodies.

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human 5-HT3-B sequence but will be capable of hybridizing to human 5-HT3-B DNA even in the presence of DNA oligonucleotides with mismatches under appropriate conditions. Under alternate conditions, the mismatched DNA oligonucleotides may still hybridize to the human 5-HT3-B DNA to permit identification and isolation of human 5-HT3-B encoding DNA.

DNA encoding human 5-HT3-B from a particular organism may be used to isolate and purify homologues of human 5-HT3-B from other organisms. To accomplish this, the first human 5-HT3-B DNA may be mixed with a sample containing DNA encoding homologues of human 5-HT3-B under appropriate hybridization conditions. The hybridized DNA complex may be isolated and the DNA encoding the homologous DNA may be purified therefrom.

It is known that there is a substantial amount of redundancy in the various codons that code for specific amino acids. Therefore, this invention is also directed to those DNA sequences that contain alternative codons that code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein that does not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally

occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of human 5-HT3-B is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of human 5-HT3-B. The term "functional derivatives" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" or to "chemical derivatives" of human 5-HT3-B. The term "fragment" is meant to refer to any polypeptide subset of human 5-HT3-B. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire human 5-HT3-B molecule or to a fragment thereof. A molecule is "substantially similar" to human 5-HT3-B if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical. The term "analog" refers to a molecule substantially similar in function to either the entire human 5-HT3-B molecule or to a fragment thereof.

Following expression of human 5-HT3-B in a recombinant host cell, human 5-HT3-B protein may be recovered to provide human 5-HT3-B in active form. Several serotonin 5-HT3-A receptor purification procedures are available and suitable for use (Fletcher and Barnes, 1997; Fletcher et al., 1998; Lummis and Martin, 1992; Miller et al., 1992). As described above for purification of human 5-HT3-B from natural sources, recombinant human 5-HT3-B may be purified from cell lysates and extracts, or from conditioned culture medium, by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography.

In addition, recombinant human 5-HT3-B can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for full length nascent human 5-HT3-B, polypeptide fragments of human 5-HT3-B or human 5-HT3-B subunits.

Monospecific antibodies to human 5-HT3-B are purified from mammalian antisera containing antibodies reactive against human 5-HT3-B or are prepared as monoclonal antibodies reactive with human 5-HT3-B using the technique of Kohler and Milstein, *Nature* 256: 495-497 (1975). Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for human 5-HT3-B. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the human 5-HT3-B, as described above. Human 5-HT3-B specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits being preferred, with an appropriate concentration of human 5-HT3-B either with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of peptide encoding a fragment of human 5-HT3-B associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing Corynebacterium parvum and tRNA. The initial immunization consists of human 5-HT3-B peptide in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of the antigen in Freund's incomplete adjuvant by the same route. Booster injections are given at about three-week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single

immunization, the animals are bled, the serum collected, and aliquots are stored at about -20°C.

Monoclonal antibodies (mAb) reactive with human 5-HT3-B are prepared by immunizing inbred mice, preferably Balb/c, with human 5-HT3-B peptide. The mice are immunized by the IP or SC route with about 0.1 mg to about 10 mg, preferably about 1 mg, of human 5-HT3-B peptide in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed above. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 0.1 to about 10 mg of human 5-HT3-B polypeptide in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions that will allow the formation of stable hybridomas. Fusion partners may include, but are not limited to: mouse myelomas P3/NS1/Ag 4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being generally preferred. The antibody producing cells and myeloma cells are fused in polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days 14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using human 5-HT3-B peptide as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a techniques such as the soft agar technique MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

Monoclonal antibodies are produced *in vivo* by injection of pristane primed Balb/c mice, approximately 0.5 ml per mouse, with about 2 x 10^6 to about 6 x 10^6 hybridoma cells about 4 days after priming. Ascites fluid is collected at approximately 8-12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of anti- human 5-HT3-B mAb is carried out by growing the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of human 5-HT3-B in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the above described methods for producing monospecific antibodies may be utilized to produce antibodies specific for human 5-HT3-B polypeptide fragments, or full-length nascent human 5-HT3-B polypeptide, or the individual human 5-HT3-B subunits. Specifically, it is readily apparent to those skilled in the art that monospecific antibodies may be generated which are specific for only the human 5-HT3-B subunit or the fully functional receptor.

Human 5-HT3-B antibody affinity columns can be made by adding the antibodies to Affigel-10 (Bio-Rad), a gel support which is activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activate esters are then quenched with ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3)

and the cell culture supernatants or cell extracts containing human 5-HT3-B subunits are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A_{280}) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6). The purified human 5-HT3-B protein is then dialyzed against phosphate buffered saline.

DNA clones, termed p5HT3BR, are identified which encode proteins that, when expressed in any recombinant host, including but not limited to mammalian cells or insect cells or bacteria form a human 5-HT3-B sensitive to serotonin when co-expressed with 5-HT3-A receptor subunits. The expression of human 5-HT3-B DNA results in the reconstitution of the properties observed in oocytes injected with human 5-HT3-B -encoding poly $(A)^+$ RNA together with 5-HT3-A receptor subunits. These include: modification of the 5-HT-, mCPBG and 1-PBG-induced responses compared to those observed for 5-HT3-A homomultimers.

Serotonin is a biogenic amine transmitter that functions in some capacity in many physiological and pathophysiological conditions. Serotonin acts as a neurotransmitter and neuromodulator in the central and peripheral nervous systems, mediates inflammatory and allergic responses, regulates airway function, controls acid secretion in the stomach, regulates cardiovascular function as well as arterial and venous responses and is likely involved in to processes yet to be determined. The serotonin receptors that mediate these include the ligand-gated 5-HT3 receptor. Overlap of 5-HT3-A and 5-HT3-B receptor expression suggests that the putative heteromultimer is involved in central and peripheral nervous system as well as small intestine, thymus, prostate and uterine function. One way to understand which serotonin receptors are involved in these processes is to develop chemical modulators of the receptors as research tools and therapeutic entities. Recombinant host cells expressing the human serotonin 5-HT3-A and human 5-HT3-B receptors can be used to provide materials for a screening method to identify such agonists and antagonists. As such, this invention of the human serotonin 5-HT3-B subunit directly teaches a way to identify new agonists

and antagonists that may prove useful as research tools or may be used as therapeutics to treat disorders directly or indirectly involving serotonin receptors.

The present invention is also directed to methods for screening for compounds that modulate the expression of DNA or RNA encoding human 5-HT3-B as well as the function of human 5-HT3-B protein *in vivo*. Compounds that modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding human 5-HT3-B, or the function of human 5-HT3-B protein. Compounds that modulate the expression of DNA or RNA encoding human 5-HT3-B or the function of human 5-HT3-B protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Modulators identified in this process are useful as therapeutic agents, research tools and diagnostic agents.

Kits containing human 5-HT3-B DNA or RNA, antibodies to human 5-HT3-B, or human 5-HT3-B protein may be prepared. Such kits are used to detect DNA that hybridizes to human 5-HT3-B DNA or to detect the presence of human 5-HT3-B protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic analyses, diagnostic applications, and epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of human 5-HT3-B DNA, human 5-HT3-B RNA or human 5-HT3-B protein. The recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of human 5-HT3-B. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant human 5-HT3-B protein or anti- human 5-

HT3-B antibodies suitable for detecting human 5-HT3-B. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Nucleotide sequences that are complementary to the human 5-HT3-B encoding DNA sequence can be synthesized for antisense therapy. These antisense molecules may be DNA, stable derivatives of DNA such as phosphorothioates or methylphosphonates, RNA, stable derivatives of RNA such as 2'-*O*-alkylRNA, or other human 5-HT3-B antisense oligonucleotide mimetics. Human 5-HT3-B antisense molecules may be introduced into cells by microinjection, liposome encapsulation or by expression from vectors harboring the antisense sequence. Human 5-HT3-B antisense therapy may be particularly useful for the treatment of diseases where it is beneficial to reduce human 5-HT3-B activity.

Human 5-HT3-B gene therapy may be used to introduce human 5-HT3-B into the cells of target organisms. The human 5-HT3-B gene can be ligated into viral vectors that mediate transfer of the human 5-HT3-B DNA by infection of recipient host cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, poliovirus and the like. Alternatively, human 5-HT3-B DNA can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted DNA transfer using ligand-DNA conjugates or adenovirus-ligand-DNA conjugates, lipofection membrane fusion or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* human 5-HT3-B gene therapy. Human 5-HT3-B gene therapy may be particularly useful for the treatment of diseases where it is beneficial to elevate human 5-HT3-B activity.

Pharmaceutically useful compositions comprising human 5-HT3-B DNA, human 5-HT3-B RNA, or human 5-HT3-B protein, or modulators of human 5-HT3-B receptor activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical

Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders in which modulation of human 5-HT3-B -related activity is indicated. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties that are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages defined by routine testing in order to obtain optimal inhibition of the human 5-HT3-B receptor or its activity while minimizing any potential toxicity. In addition, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds or modulators identified according to this invention as the active ingredient for use in the modulation of human 5-HT3-B receptors can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds or modulators can be administered in such oral dosage forms as tablets, capsules (each including timed

release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. An effective but non-toxic amount of the compound desired can be employed as a serotonin 5-HT3-A/5-HT3-B receptor modulating agent.

The daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per patient, per day. For oral administration, the compositions are preferably provided in the form of scored or unscored tablets containing 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, and 50.0 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.0001 mg/kg to about 100 mg/kg of body weight per day. The range is more particularly from about 0.001 mg/kg to 10 mg/kg of body weight per day. The dosages of the human 5-HT3-B receptor modulators are adjusted when combined to achieve desired effects. On the other hand, dosages of these various agents may be independently optimized and combined to achieve a synergistic result wherein the pathology is reduced more than it would be if either agent were used alone.

Advantageously, compounds or modulators of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds or modulators for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds or modulators of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

In the methods of the present invention, the compounds or modulators herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like.

Lubricants used in these dosage forms include, without limitation, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methylcellulose, agar, bentonite, xanthan gum and the like.

For liquid forms the active drug component can be combined in suitably flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methyl-cellulose and the like. Other dispersing agents that may be employed include glycerin and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations, which generally contain suitable preservatives, are employed when intravenous administration is desired.

Topical preparations containing the active drug component can be admixed with a variety of carrier materials well known in the art, such as, e.g., alcohols, aloe vera gel, allantoin, glycerine, vitamin A and E oils, mineral oil, PPG2 myristyl propionate, and the like, to form, e.g., alcoholic solutions, topical cleansers, cleansing creams, skin gels, skin lotions, and shampoos in cream or gel formulations.

The compounds or modulators of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

Compounds of the present invention may also be delivered by the use of monoclonal antibodies as individual carriers to which the compound molecules are coupled. The compounds or modulators of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropylmethacryl-amidephenol, polyhydroxy-ethylaspartamidephenol, or polyethyl-eneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds or modulators of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for

example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydro-pyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

For oral administration, the compounds or modulators may be administered in capsule, tablet, or bolus form or alternatively they can be mixed in the animals feed. The capsules, tablets, and boluses are comprised of the active ingredient in combination with an appropriate carrier vehicle such as starch, talc, magnesium stearate, or di-calcium phosphate. These unit dosage forms are prepared by intimately mixing the active ingredient with suitable finely-powdered inert ingredients including diluents, fillers, disintegrating agents, and/or binders such that a uniform mixture is obtained. An inert ingredient is one that will not react with the compounds or modulators and which is non-toxic to the animal being treated. Suitable inert ingredients include starch, lactose, talc, magnesium stearate, vegetable gums and oils, and the like. These formulations may contain a widely variable amount of the active and inactive ingredients depending on numerous factors such as the size and type of the animal species to be treated and the type and severity of the infection. The active ingredient may also be administered as an additive to the feed by simply mixing the compound with the feedstuff or by applying the compound to the surface of the feed. Alternatively the active ingredient may be mixed with an inert carrier and the resulting composition may then either be mixed with the feed or fed directly to the animal. Suitable inert carriers include corn meal, citrus meal, fermentation residues, soya grits, dried grains and the like. The active ingredients are intimately mixed with these inert carriers by grinding, stirring, milling, or tumbling such that the final composition contains from 0.001 to 5% by weight of the active ingredient.

The compounds or modulators may alternatively be administered parenterally via injection of a formulation consisting of the active ingredient dissolved in an inert liquid carrier. Injection may be either intramuscular,

intraruminal, intratracheal, or subcutaneous. The injectable formulation consists of the active ingredient mixed with an appropriate inert liquid carrier. Acceptable liquid carriers include the vegetable oils such as peanut oil, cottonseed oil, sesame oil and the like as well as organic solvents such as solketal, glycerol formal and the like. As an alternative, aqueous parenteral formulations may also be used. The vegetable oils are the preferred liquid carriers. The formulations are prepared by dissolving or suspending the active ingredient in the liquid carrier such that the final formulation contains from 0.005 to 10% by weight of the active ingredient.

Topical application of the compounds or modulators is possible through the use of a liquid drench or a shampoo containing the instant compounds or modulators as an aqueous solution or suspension. These formulations generally contain a suspending agent such as bentonite and normally will also contain an antifoaming agent. Formulations containing from 0.005 to 10% by weight of the active ingredient are acceptable. Preferred formulations are those containing from 0.01 to 5% by weight of the instant compounds or modulators.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1:

Cloning of p5HT3BR

cDNA synthesis

First strand synthesis: Approximately 5 μg of human small intestine mRNA (Clontech) was used to synthesize cDNA using the cDNA synthesis kit (Life Technologies). 2 μl of NotI primer adapter was added to 5μl of mRNA and the mixture was heated to 70 ^{0} C for 10 minutes and placed on ice. The following reagents were added on ice: 4μl of 5x first strand buffer (250mM TRIS-HCl (pH8.3), 375mM KCl, 15mMMgCl_2), 2μl of 0.1M DTT, 10mM dNTP (nucleotide

triphosphates) mix and 1µl of DEPC treated water. The reaction was incubated at 42°C for 5minutes. Finally, 5µl of Superscript RT II was added and incubated at 42°C for 2 more hours. The reaction was terminated on ice.

Second strand synthesis: The first strand product was adjusted to 93µl with water and the following reagents were added on ice: 30 µl of 5x 2nd strand buffer (100 mM TRIS-HCl (pH6.9), 450 mM KCl, 23 mM $MgCl_2$, 0.75 mM β-NAD+, 50mM $(NH_4)_2SO_4$), 3µl of 10 mM dNTP (nucleotide triphosphates), 1µl E. coli DNA ligase (10units) 1µl RNase H (2units), 4 µl DNA pol I (10 units). The reaction was incubated at 16°C for 2 hours. The DNA from second strand synthesis was treated with T4 DNA polymerase and placed at 16°C to blunt the DNA ends. The double stranded cDNA was extracted with 150 µl of a mixture of phenol and chloroform (1:1, v:v) and precipitated with 0.5 volumes of 7.5 M NH4OAc and 2 volumes of absolute ethanol. The pellet was washed with 70% ethanol and dried down at 37°C to remove the residual ethanol. The double stranded DNA pellet was resuspended in 25 µl of water and the following reagents were added; 10 µl of 5x T4 DNA ligase buffer, 10 µl of Sal1 adapters and 5 µl of T4 DNA ligase. The ingredients were mixed gently and ligated overnight at 16° C. The ligation mix was extracted with phenol:chloroform:isoamyl alcohol, vortexed thoroughly and centrifuged at room temperature for 5 minutes at 14,000 x g to separate the phases. The aqueous phase was transferred to a new tube and the volume adjusted to 100 ml with water. The purified DNA was size selected on a chromaspin 1000 column (Clontech) to eliminate the smaller cDNA molecules. The double stranded DNA was digested with NotI restriction enzyme for 3-4 hours at 37° C. The restriction digest was electrophoresed on a 0.8 % low melt agarose gel. The cDNA in the range of 1-5 KB was cut out and purified using Gelzyme (InVitrogen). The product was extracted with phenol:chloroform and precipitated with NH_4OAc and absolute ethanol. The pellet was washed with 70% ethanol and resuspended in 10 ml of water.

Ligation of cDNA to the Vector: The cDNA was split up into 5 tubes (2μl each) and the ligation reactions were set up by adding 4.5 μl of water, 2 μl of 5x ligation buffer, 1μl of p-Sport vector DNA (cut with Sal-1 / NotI and phosphatase treated) and 0.5 μl of T4 DNA ligase. The ligation was incubated at 40° C overnight.

Introduction of Ligated cDNA into E. coli by Electroporation:
The ligation reaction volume was adjusted to a total volume of 20 μl with water. Five ml of yeast tRNA, 12.5 ml of 7.5M NH_4OAc and 70 ml of absolute ethanol (-20°C) was added. The mixture was vortexed thoroughly, and immediately centrifuged at room temperature for 20 minutes at 14,000 x g. The pellets were washed in 70% ethanol and each pellet was resuspended in 5 ml of water. All 5 ligations (25ml) were pooled and 100μl of DH10B electro-competent cells (Life Technologies) were electroporated with 1 ml of DNA (total of 20 electroporations), then plated out on ampicillin plates to determine the number of recombinants (cfu) per μl. The entire library was seeded into 2 liters of Super Broth and maxipreps were made using Promega Maxi Prep kit and purified on cesium chloride gradients.

Screening of library:

One-microliter aliquots of the library constructed above were electroporated into Electromax DH10B cells (Life Technologies). The volume was adjusted to 1 ml with SOC media and incubated for 1 hour at 37°C with shaking. The library was then plated out on 50 150cm^2 plates containing LB to a density of 5000 colonies per plate. These were grown overnight at 37°C.

A probe to 5-HT3-B was generated by polymerase chain reaction using the following primer pair:

5' oligo: 5' GAT CTC CCT ACC TCT AAG TG 3' {SEQ.ID. NO.: 1]

3' oligo: 5' AGC ACA CTG GTC TTG AAC AC 3' [SEQ.ID.NO. 2].

Amplification was cycled 35 times using 50-60°C annealing temperature and a human small intestine cDNA as template. The PCR fragment that was generated (400-500 bp) was 32P-labelled using the Klenow fragment of DNA polymerase I and an oligo labeling kit (Pharmacia). The fragment was then cleaned by one passage through a S-200 column (Pharmacia).

The library colonies are lifted on nitrocellulose filters and cross-linked via UV irradiation (Stratagene). Filters were washed three times in buffer (50 mM TRIS, 1 M NaCl, 2mM EDTA, 1% SDS) at 42°C. Filters were then prehybridized in 1:1 Southern Prehyb:Formamide with salmon sperm DNA (50mg, boiled) for 6 hours at 42°C. Filters were then hybridized with the probe ($1x10^6$ counts/ml) overnight. The filters were then washed one time with 2xSSC/0.2%SDS at room temperature for 15 minutes, 2 times with 0.2xSSC/0.1%SDS at 45°C for 30 minutes each. Filters were then wrapped in plastic wrap and exposed to film (Kodak) overnight at -80°C.

Positive clones were identified. Resulting positives were cored from the original plate, incubated in LB for 45 minutes at 37°C and re-plated overnight. The filter lifting/hybridizing/washing/colony picking procedure was replicated until a single clone or clones were isolated, representing an individual cDNA.

From the screen for human novel 5HT3-like receptor, all cDNA clones were isolated and sequenced. One clone, pH3R, contained a 2699 bp insert (Figure 1). This sequence had an apparent open reading frame from nucleotide 299 to 1335 (Figure 2). This open reading frame encoded a protein of 445 amino acids (Figure 3).

EXAMPLE 2

Cloning of 5-HT3-B cDNA into a Mammalian Expression Vector

The 5-HT3-B cDNAs (collectively referred to as p5HT3BR) were cloned into the mammalian expression vector pcDNA3.1zeo(+) (InVitrogen). The 5-

HT3-B cDNA clone was isolated from a human small intestine cDNA library. The full-length cDNA was used as the template for PCR using specific primers with BamHI (5'AAC GTT GAA TTC GCC ACC ATG TTG TCA AGT GTA ATG GCT CCC CTG TGG GCC3') [SEQ.ID.NO. 3] and HindIII (5'AAC GTT AAG CTT TCT TAA GTG CCA GCA CAA TTA CTT GAA G 3') [SEQ.ID.NO. 4] sites for cloning. The PCR product was purified on a column (Wizard PCR DNA purification kit from Promega) and digested with NheI and NotI (NEB) to create cohesive ends. The product was purified by a low melting agarose gel electrophoresis. The pcDNA3.1zeo(+) vector was digested with NheI and NotI enzymes and subsequently purified on a low melt agarose gel. The linear vector was used to ligate to the 5-HT3-B cDNA inserts. Recombinants were isolated, designated 5-HT3-B, and used to transfect mammalian cells stably expressing the human 5-HT3-A receptor transfected in a pCIneo vector (using EcoRI and XbaI cloning sites) (5-HT3-A/HEK293 cells) by electroporation. Stable cell clones were selected by growth in the presence of G418 and zeocin. Single G418/zeocin resistant clones were isolated and shown to contain the intact 5-HT3-B gene. Clones containing the human 5-HT3-B cDNAs were analyzed for p5HT3BR expression by measuring Ca^{2+} influx using Fluo-4 in response to serotonin and 1-PBG (Figure 9 a, b, c). Responses were compared to those obtained from 5-HT3-A-expressing HEK293 cells.

Cells stably expressing human 5-HT3-B together with human 5-HT3-A were used to test for expression human 5-HT3-B and for functional activity. These cells are used to identify and examine other compounds for their ability to modulate, inhibit or activate the human 5-HT3-B. Other cells expressing both 5-HT3-A and 5-HT3-B subunits can be used to identify and examine other compounds for their ability to modulate, inhibit or activate the human 5-HT3-B.

Cassettes containing the human 5-HT3-B cDNA in the positive orientation with respect to the promoter are ligated into appropriate restriction sites 3' of the promoter and identified by restriction site mapping and/or sequencing. These cDNA expression vectors are introduced into fibroblastic host cells for example

HEK293 by standard methods including but not limited to electroporation, or chemical procedures (cationic liposomes, DEAE dextran, calcium phosphate).

All of the vectors used for mammalian transient expression can be used to establish stable cell lines expressing the human 5-HT3-B. Unaltered human 5-HT3-B constructs cloned into expression vectors are expected to program host cells to make human 5-HT3-B protein. The transfection host cells include, but are not limited to, HEK293, CV-1-P [Sackevitz et al., Science 238: 1575 (1987)], tk-L [Wigler, et al. Cell 11: 223 (1977)], NS/0, and dHFr- CHO [Kaufman and Sharp, J. Mol. Biol. 159: 601, (1982)].

Co-transfection of any vector containing the human 5-HT3-B cDNA with a drug selection plasmid including, but not limited to G418, zeocin, aminoglycoside phosphotransferase; hygromycin, hygromycin-B phosphotransferase; APRT, xanthine-guanine phosphoribosyl-transferase, will allow for the selection of stably transfected clones. Levels of the human 5-HT3-B are quantitated by the assays described herein.

The human 5-HT3-B cDNA constructs are also ligated into vectors containing amplifiable drug-resistance markers for the production of mammalian cell clones synthesizing the highest possible levels of the human 5-HT3-B. Following introduction of these constructs into cells, clones containing the plasmid are selected with the appropriate agent, and isolation of an over-expressing clone with a high copy number of plasmids is accomplished by selection in increasing doses of the agent.

The expression of recombinant human 5-HT3-B is achieved by transfection of full-length the human 5-HT3-B cDNA into a mammalian host cell.

EXAMPLE 3

Primary Structure of the Human 5-HT3-B Protein

The nucleotide sequences of p5HT3BR revealed single large open reading frame of about 1326 base pairs as shown in Figure 2. The cDNAs have 5' and 3'-untranslated extensions of about 141 and about 456 nucleotides for p5-HT3-BR.

The first in-frame methionine was designated as the initiation codon for an open reading frame that predicts a human 5-HT3-B protein with an estimated molecular mass (M_r) of about 50.3 kDa. The protein contained hydrophobic amino terminal residues with sequences highly predictive of signal cleavage sites that would result in mature proteins initiating at amino acid 22.

The predicted human 5-HT3-B protein was aligned with nucleotide and protein databases and found to be related to the known 5-HT3-A receptors. Approximately 70% of the amino acids in 5-HT3-B were highly conserved, showing at least 44% amino acid identity within the serotonin 5-HT3 family of receptor. The conserved motifs found in this family of receptor, such as the 4 putative transmembrane domains with similar spacing, were also found in the human 5-HT3-B sequence. The identity of the 5-HT3-B receptor with the 5-HT3-A receptor at the nucleotide level was only about 60%. The human 5-HT3-B protein contained the conserved cysteine residues found in the conserved cysteine-cysteine loop that may form the agonist-binding site of ligand-gated ion channels (Lambert et al., 1995). There is strong homology to the proposed ligand recognition site in the first N-terminal loop in the murine 5-HT3-A and the nicotinic AChR α7 [xIWxPDILxxExxD]; the only difference in the shown "consensus" in the 5-HT3-B protein is a conserved change: L119 to I119. The E106 in the 5-HT3-A (murine) is critical for high affinity 5-HT binding (Boess et al., 1997).

Five potential sites of glycosylation (Marshall, 1972) were located at the extracellular amino terminus and 1 potential site for protein kinase C (Woodgett et la., 1986), 3 potential sites for casein kinase II (Pinna, 1990), and 1 site for mammary gland casein kinase were located in the cytoplasmic loop between M3 and M4 as shown in Figure 3.

EXAMPLE 4

Distribution of 5-HT3-B mRNA.

The tissue distribution of 5-HT3-B mRNAs was determined by semi-quantitative PCR. A primer set specific to 5-HT3-B (TGTGTTCAAGACCAGTGTGC [SEQ.ID.NO.8]; TAGCTTTGGAAGAGCAGTCG [SEQ.ID.NO.9]) was used to complete amplification of a portion of the 5-HT3-B mRNA via PCR using cDNAs templates synthesized from poly (A) RNA (Clontech, Palo Alto, CA) which was extracted from various human tissues (tissue types shown in Figure 4a). To gain increased specificity and sensitivity, an oligonucleotide (TGTTGGTCAAATTCCTCCATGATGAGCAGCGTGGTGGACA [SEQ.ID.NO.10]) was phosphorylated using γ-^{32}P-ATP with polynucleotide kinase as described by manufacturer (Amersham Pharmacia Biotech, Piscataway, New Jersey) and annealed to denatured PCR products and resolved by 6% polyacrylamide gel electrophoresis. The subsequent gel was then dried down and imaged (PhosphorImager 445SI, Molecular Dynamics).

As shown in figure 4a, PCR-based tissue distribution analysis reveals that the 5-HT3-B mRNA is expressed in human cerebral cortex including occipital, frontal and temporal regions, amygdala, hippocampus, testis. Very low levels were observed in adrenal gland, bone marrow, lymph node, salivary gland, thyroid. No detectable transcript was observed in heart, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, ovary, small intestine, colon, leukocytes, cerebellum, medulla, spinal cord, putamen, caudate nucleus, corpus callosum, substantia nigra, and thalamus.

In the CNS, the 5-HT3 receptor is found in high density in nuclei of the lower brainstem, area postrema and nucleus of the tractus solitarius. Lower densities of the receptor are found in the cerebral cortex and limbic areas, including the hippocampus. In the periphery, 5-HT3 receptors are located on pre- and postganglionic neurons of both sensory and enteric nervous systems (Eglen

and Bonhaus, 1996). Northern analysis revealed some overlap of 5-HT3-B and 5-HT3-A receptor distributions.

Figure 4b shows RT-PCR in situ hybridization that was performed on normal human tissues of the spleen (b1, b2) and small intestine (b3, b4). Human spleen had intense 5-HT3-B mRNA (b1) and 5HT3-A mRNA (b2) signal (purple in color – dark grey in black-and-white), respectively, in small lymphocytes (arrows). Similarly, intense 5-HT3-B mRNA (b3) and 5HT3-A mRNA (b4) signal (purple) was detected in stromal fibroblasts of the small intestines (arrows). Total magnification = 600x.

Figure 4c shows RT-PCR in situ hybridization that was performed on serial 10 μm sections of the normal monkey amygdala. Figures 4c1 (300x) and 4c3 (600x) show positive 5-HT3-A mRNA signal (purple in color – dark grey in black-and-white) in seven neurons (arrows) which is also show positive 5HT3-B mRNA signal (4c2 (300x); 4c4 (600x)). Not all cells were labeled by the RT-PCR ISH technique using specific probes for 5-HT3-B or 5-HT3-A.

EXAMPLE 5

Characterization of the Function of protein encoded by p5HT3BR in Xenopus oocytes

Xenopus laevis oocytes were prepared and injected using standard methods previously described and known in the art (Fraser et al., 1993). Ovarian lobes from adult female *Xenopus laevis* (Nasco, Fort Atkinson, WI) were teased apart, rinsed several times in nominally Ca-free saline containing (in mM): NaCl 82.5, KCl 2.5, $MgCl_2$ 1, HEPES 5, adjusted to pH 7.0 with NaOH (OR-2), and gently shaken in OR-2 containing 0.2% collagenase Type 1 (ICN Biomedicals, Aurora, Ohio) for 2-5 hours. When approximately 50% of the follicular layers were removed, Stage V and VI oocytes were selected and rinsed in media consisting of 75% OR-2 and 25% ND-96. The ND-96 contained (in mM): NaCl 100, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, Na pyruvate 2.5, gentamicin (50 ug/ml), adjusted to pH 7.0 with NaOH. The extracellular Ca^{2+} was gradually increased and the cells were maintained in ND-96 for

2-24 hours before injection. For in vitro transcription, pGEM HE (Liman et al., 1992)) containing human 5-HT3-A (Genbank D49394) or 5-HT3-B cDNA was linearized with NheI and transcribed with T7 or SP6 RNA polymerase (Stratagene) in the presence of the cap analog m7G(5')ppp(5')G. The synthesized cRNA was purified with a Sephadex G-50 spin column. Oocytes were injected with 50 nl of the human serotonin 5-HT3-A receptor with or without the 5-HT3-B RNA (0.02 and 0.002-0.2 ng each) or other channel or receptor subunit. Control oocytes were injected with 50 nl of water. Oocytes were incubated for 2-10 days in ND-96 before analysis for expression of the human 5-HT3-B. Incubations and collagenase digestion were carried out at room temperature. Injected oocytes were maintained in 48 well cell culture clusters (Costar, Cambridge, MA) at 18°C. Whole cell agonist-induced currents were measured 1-14 days after injection with a conventional two-electrode voltage clamp (GeneClamp500, Axon Instruments, Foster City, CA) using standard methods previously described and known in the art (Dascal, 1987). The microelectrodes were filled with 3 M KCl, which had resistances of 1 and 2 MΩ. Cells were continuously perfused with ND96 at 10 ml/min at room temperature unless indicated. Membrane voltage was clamped at -88 mV unless indicated.

5-HT (≥100 μM) had no effect in oocytes injected with putative 5-HT3-B subunit alone (n= 4 (3.3 ng cRNA/oocyte), n= 12 (0.33 ng), n= 12 (0.033 ng), and n=3 (0.0033 ng)) indicating there were no endogenous 5-HT-induced currents in the oocytes used in these studies. Oocytes injected with only 5-HT3-B cRNA (3.3 ng) or 5-HT3-B together with nACh β1, β2 and β3 were insensitive to 300 neuroactive compounds at ≥ 100 μM including 5-HT, ACh, histamine, tyramine, tryptamine, tryptophanamide, tryptophan, norepinephrine, octopamine, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid, tryptophol, alpha-methyl serotonin, glutamate, glycine, GABA, β-alanine, taurine, β-phenylethylamine, 5-hydroxyindolacetic acid, 5-hydroxyindole, 6-hydroxymelatonin, gamma hydroxybutyrate, cis-4 aminocrotonic acid, agmatine, d-cycloserine, N-acetyl-L-cysteine, acetyl-aspartyl-L-glutamic acid, S-α-histamine, N-α-methyl histamine,

melatonin, 5-hydroxyindole 2-carboxylic acid, N-acetyl serotonin, and 5-hydroxyindole 3-acetamide. Injection of 5-HT3-B-injected oocytes with bacterial alkaline phosphatase (0.25- 0.3 U) at least 30 min prior to recording was ineffective in conferring sensitivity to these ligands (n=3).

Three characteristics of the 5-HT3-A receptor agonist-induced responses were dramatically altered in 5-HT3-B-injected oocytes. First, the kinetics of the 5-HT-induced response were markedly altered (Figure 5a); second, peak currents were increased (Figure 5c); and third, 5-HT3-B specifically modified the dose-response relationship to 5-HT, the biphenylguanide derivatives mCPBG and 1-PBG, and 2-Me-5-HT, with no detectable effect on the dose-response to dopamine (DA).

Functional expression of human serotonin 5-HT3 receptor modifier subunit 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes is shown in Figure 5. Oocytes were continuously perfused with Ba^{2+} containing ND96 at a rate of 10 ml/min at room temperature. The inward current through 5-HT3-A homomeric receptors declined during the continued presence of 1 to 100 μM 5-HT as shown in Figure 5a, consistent with the desensitization previously described for this receptor (Belelli et al., 1995; Hope et al., 1996; Lankiewicz et al., 1998). 5-HT3-A-expressing oocytes responded to 5-HT, 2-methyl-5-HT, mCPBG and 1-PBG with either a slowly desensitizing response (left panel; "slow" responders, 71% of oocyte batches), or a complex current that included a rapidly desensitizing component (right panel; "fast" responders). Oocytes expressing both subunits produced uniform responses (middle panel). The agonists tested included 5-HT (0.3 and 10 μM, responses are superimposed); mCPBG (0.3 and 10 μM); 1-PBG (10 and 100 μM); DA (0.1 and 1 mM); 2-Me-5-HT (10 μM). Agonists are applied during the time indicated by the horizontal bar above the record. The clear bar extending from the solid bar indicates the longer exposure to agonist for one of the superimposed traces. Time scale bar: 40 sec.

Oocytes that had been co-injected with equivalent amounts of 5-HT3-A and 5-HT3-B cRNA always responded similarly, despite radical differences in response kinetics of the two populations of control 5-HT3-A-injected oocytes (Table 1). Thus, in "slow" oocytes, near-maximal 5-HT-induced currents decayed 5 times more quickly (Figure 5, left vs. middle panels; Figure 6a; Table 1), while in "fast" oocytes, responses decayed 3.5-fold more slowly (Figure 5, right vs. middle panels; Figure 6b; Table 1). Furthermore, the time to peak for responses to 5-HT (10 μM) was 3.3 +/- 0.3 sec (n= 38) in the presence of 5-HT3-B for all oocytes tested (Figure 6c,d, solid bars, "slow" and "fast" responders). Thus, in the presence of 5-HT3-B, 5-HT3 receptor responses in oocytes were normalized to a moderately transient current. In contrast to the complex response waveforms observed in the absence of 5-HT3-B, inward currents elicited by 10 μM 5-HT in Ba^{2+} ND96 were best fit to a single exponential (τ=4.9 +/- 0.3 sec; n=25) in more than 80% of the oocytes expressing both subunits. This decay constant was significantly slower than the decay of "fast" responses under identical conditions ($p < 5e^{-6}$). In "slow" responders expressing 5-HT3-B, 5-HT, 2-Methyl-5-HT, mCPBG and 1-PBG decreased the t80 values by 5.6-fold, 5-fold, 2.0-fold and 1.7 fold, respectively, suggesting agonist-dependent differences (Table 1). In contrast, the 5-HT3-B modulatory effect on t80 values of "fast" responders to these agonists was about four-fold. Significant differences in t80 values were observed at 1 and 10 μM 5-HT in all oocyte batches. Interestingly, DA responses did not appear to be altered by 5-HT3-B co-expression (Figure 5a).

The response of homomeric and heteromeric receptors to 5-HT was consistent with the activation of non-selective cation channels since the current reversed near 0 mV. The differences in kinetics appeared to be due to alterations in 5-HT3 receptor function and not due to activation of contaminating endogenous Ca^{2+}-activated chloride channels. While 5-HT3-A receptors are permeable to Ca^{2+} (Davies et al., 1999; Hargreaves et al., 1994; Ronde and Nichols, 1998; Yang, 1990), the influx of Ca^{2+} appears to be insufficient to activate endogenous chloride currents (Gilon and Yakel, 1995; Mair et al., 1998). Furthermore, 5-HT3-B diminishes the Ca^{2+}

permeability of the human recombinant receptor (Davies et al., 1999). 5-HT3-B modulation of channel properties were observed in salines deplete of Ca^{2+}, and at membrane potentials near the chloride equilibrium potential.

Responses to 1-PBG were blocked by the 5-HT3 antagonist tropisetron. After obtaining a control response to 1-PGB (100 μM; indicated by the solid bar) (Figure 5b, left panel), the oocyte was washed 2 min then incubated for 30 sec in tropisetron (1 μM; clear bar) then challenged with both 1-PBG and tropisetron. The response to 1-PBG was completely blocked (Figure 5b, middle panel). The response to agonist recovered after a 2 min washout of antagonist (Figure 5b, right panel).

Co-expression of 5-HT3-A together with 5-HT3-B cRNA (solid bars, Figure 5c) significantly increased the maximum response to mCPBG, 1-PBG and 5-HT compared to expression with 5-HT3-A alone (grey bars, Figure 5c). Data from oocytes recorded in Ba^{2+}- and Ca^{2+}- containing saline were combined since there were no differences in 5-HT (10 μM)-induced maximal current in Ca^{2+}-containing vs. Ba^{2+}-containing saline (-7.7 +/- 1.4 μA (n=16) vs. -7.6 +/- 0.9 μA (n=24)). The number of oocytes tested ranged from 9 to 61; SEM values were 10% for 5-HT (10 μM) and 21% for both mCPBG (10 μM) and 1-PBG (100 μM). p values are indicated: * $p<0.05$, ** $p<0.01$ (Students t-test).

The desensitization of the response was quantified by measuring the time between 80% to peak on the rising and falling phases of the response (t80). The rate of decay was slower in salines in which Ca^{2+} was replaced by Ba^{2+}: in Ca^{2+}- and Ba^{2+}-containing saline, t80 was 11.3 +/- 2.6 sec (n= 18) and 30.3 +/- 4.3 sec (n= 31; $p< 0.0005$), respectively. Under no condition tested was t80 dependent on voltage. The initial decay of the "fast" responders could be fit by a single exponential with $\tau= 2.0$ +/- 0.3 sec (n=8). Our quantification of kinetic parameters determined from oocyte currents provides a means of comparing response durations and onset between populations of oocytes treated under similar experimental conditions. It is clearly not

an accurate description of the underlying channel activity because the relatively slow method of application and oocyte geometry precludes rapid exchange of solutions.

The time to peak of the response to 5-HT (10 μM) was 6.2 +/- 0.6 sec (n=41; Figure 6c) and 1.8 +/- 0.2 sec (n=10; Figure 6d) for slow and fast responders, respectively. Similar differences were obtained in response to 100 μM 5-HT. The t80 of homomeric "fast" responses was voltage independent in both in Ca^{2+}- and Ba^{2+}-containing salines similar to the finding with "slow" responses, however, in contrast to the latter, the t80 was similar in the presence and absence of Ca^{2+}.

The pharmacology of 5-HT3 receptors was modified by 5-HT3-B expression similarly in all batches of oocytes tested and the data were combined. The apparent affinity for 5-HT was decreased when 5-HT3-B was co-expressed with 5-HT3-A in oocytes (Figure 7a). On the other hand, oocytes expressing both subunits were more sensitive to application of low concentrations of mCPBG and 1-PBG compared to oocytes expressing 5-HT3-A alone (Figure 5a). Responses to low concentrations of DA were not enhanced by 5-HT3-B. When ratios of peak response to low and high concentrations of agonist were determined, co-injected oocytes had a larger relative response to the biphenylguanide derivatives (1-PBG and mCPBG) but smaller relative response to 5-HT and the magnitude of these differences depended on the relative ratio of cRNAs injected (Figure 7b). Consistent with the t80 value dependence on ratios of injected cRNA, the differences in pharmacology were no longer observed when 5-HT3-B cRNA was 100-fold more dilute than 5-HT3-A cRNA.

The agonists 1-PBG and mCPBG elicited no response in oocytes injected with 0.33 ng 5-HT3-B subunit alone (the concentration injected to give a 1:1 ratio; n=4), indicating that the increase in 1-PBG and mCPBG responsiveness in co-injected oocytes was not due to a direct activation of 5-HT3-B homomultimers by these agonists. Furthermore, the response to 1-PBG was similarly blocked by tropisetron (1 μM), LY-278,584 maleate (1 μM), d-tubocurarine (30 μM) in a reversible manner. The selective 5-HT_2 receptor antagonist ketanserin (10 μM) had no effect on agonist

responses. The 5-HT-induced response in co-injected oocytes was also blocked by these antagonists.

However, 5-HT3-B altered the voltage-dependence of agonist-induced currents such that they were linear rather than inward rectifying. In experiments in which the currents though 5-HT3 receptors were measured at a range of membrane voltages, 5-HT3-A receptors passed more inward current than outward current at voltages negative and positive to the reversal potential, respectively. In the presence of 5-HT3-B, inward and outward currents were similar and the current-voltage relationship was nearly linear. Injection of the 5-HT3-B RNA had no effect on currents through nACh receptors expressed in oocytes (Figure 8) as well as a *Shaker* potassium channel mutant lacking the N-terminal domain responsible for fast inactivation.

The specificity of the modulatory effect of human 5-HT3-B in oocytes is shown in Figure 8. In all cases the amount of injected 5-HT3-B cRNA was at least 2-fold the concentration of each nACh receptor. (a.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α3β4 with (solid; n= 5) and without (grey; n=4) 5-HT3-B. (b.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α4β2 with (solid; n=8) and without (grey; n=9) 5-HT3-B. (c.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α2β2 with (solid; n=3) and without (grey; n=3) 5-HT3-B. (d.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α7 with (solid; n=12) and without (grey; n=15) 5-HT3-B.

Furthermore, the responses to low and high concentrations of epibatidine (0.3 and 10 μM) were similar in α7 nACh-injected oocytes in the presence or absence of 5-HT3-B indicating that the dose response relationship was not appreciably altered.

EXAMPLE 6

Characterization of the Human 5-HT3-B

Human HEK293 cells stably expressing the human 5-HT3-A receptor were transfected with p5HT3BR. One double transfectant was similar to control 5-HT3-A/HEK cells and was usually used as a control. After three days the cells were selected in the presence of neomycin (500 μg/ml) and zeocin (200 μg/ml) and grown through three 1:10 splits for approximately two weeks. Individual colonies were picked and grown in 6-well dishes. Cells were then plated onto 96-well plates (Biocoat, poly-D-lysine coated black/clear plate, Becton Dickinson part # 354640) and grown to confluence for three days. Wells were rinsed with F12/DMEM, then incubated in Fluo-4 (2 μM) with Pluronic acid (20%, 40μl used in 20 ml total volume) for 1 hour at room temperature. Plates were assayed using the FLIPR (Molecular Devices, FL-101). Cells were challenged with agonists (at 3-fold concentration in 40 μl added to 80 μl at a velocity of 50 μl/sec).

The whole cell patch clamp technique (Hamill et al., 1981) was used to record ligand-induced currents from HEK293 stably expressing 5-HT3-A receptor or both the 5-HT3-A receptor and the 5-HT3-B protein maintained for >2 days on 12 mm coverslips. Cells were visualized using a Nikon Diaphot 300 with DIC Nomarski optics. Cells were continuously perfused in a physiological saline (~0.5 ml/min) unless otherwise indicated. The standard physiological saline ("Tyrodes") contained (in mM): 130 NaCl, 4 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, and 10 hemi-Na-HEPES (pH 7.3, 295-300 mOsm as measured using a Wescor 5500 vapor-pressure (Wescor, Inc., Logan, UT). Recording electrodes were fabricated from borosilicate capillary tubing (R6; Garner Glass, Claremont, CA), the tips were coated with dental periphery wax (Miles Laboratories, South Bend, IN), and had resistances of 1-2 MΩ when containing intracellular saline (in mM: 100 K-gluconate, 25 KCl, 0.483 $CaCl_2$, 3 $MgCl_2$, 10 hemi-Na-HEPES and 1 K_4-BAPTA (100nM free Ca^{2+}); pH 7.4, with dextrose added to achieve 290 mOsm). Liquid junction potentials were -18 mV using standard pipette

and bath solutions as determined both empirically and using the computer program JPCalc (Barry, 1994). All voltages shown are corrected for liquid junction potential. Current and voltage signals were detected and filtered at 2 kHz with an Axopatch 1D patch-clamp amplifier (Axon Instruments, Foster City, CA), digitally recorded with a DigiData 1200B laboratory interface (Axon Instruments), and PC compatible computer system and stored on magnetic disk for off-line analysis. Data acquisition and analysis were performed with PClamp software. Slow changes in holding current were detected and filtered at 2 kHz, and recorded with a LPF202A DC amplifier (Warner, Hamden, CT) and VR-10B digital data recorder (Instrutech, Great Neck, NY) onto video tape. The signal was later analyzed at 10 Hz using Axotape software.

The total membrane capacitance (C_m) was determined as the difference between the maximum current after a 30 mV hyperpolarizing voltage ramp from -68 mV generated at a rate of 10 mV/ms and the steady state current at the final potential (-98 mV) (Dubin et al., 1999).

Apparent reversal potentials (V_{rev}) of ligand-induced conductance changes were determined using a voltage-ramp protocol (Dubin et al., 1999). Voltage ramps were applied every 1 second and the resulting whole cell ramp-induced currents were recorded. Usually the voltage was ramped from negative to positive to negative values. The current required to clamp the cells at -68 mV was continuously monitored. Ligand-induced conductances were determined from whole-cell currents elicited by a voltage-ramp protocol in the presence and absence of ligand. Comparison of control ramp currents and those obtained in the presence of ligand reveals the difference between these currents and indicates the effect of the ligand on the channel protein. The voltage at which there was no net ligand-induced current was determined (V_{rev}).

Most values are presented as the arithmetic mean +/- standard error of the mean (S.E.M.).

Agonist-induced Ca^{2+} and ionic current responses had markedly faster decay kinetics in HEK293 cells stably transfected with both 5-HT3-A and 5-HT3-B subunits

compared to cells stably expressing 5-HT3-A receptors (Figure 9). Cells expressing both 5-HT3-B and 5-HT3-A (Figure 9a, right) responded to 3 μM 5-HT (top, largest response), 30 μM 1-PBG (middle, largest response) and 3 μM mCPBG (bottom, largest response) with a faster decay compared to 5-HT3-A receptor-expressing cells (Figure 9a, left). These data are similar to those obtained from "slow" oocytes (Figure 5). Superimposed on the Ca^{2+} influx induced by high concentrations of agonist are responses of cells from the same plate to low concentrations of agonist (Figure 9a). As in the oocytes (Figure 7b), responses to low doses of mCPBG and 1-PBG were a larger percentage of the maximum response observed in doubly transfected cells (Figure 9c; $p< 0.005$ at 0.1 and 0.17 μM mCPBG). However, in contrast to the oocyte data (Figure 7a,b), low concentrations of 5-HT also elicited larger responses in double transfectants compared to 5-HT3-A/HEK (Figure 9b; $p < 0.005$ at 60, 100 and 170 nM 5-HT). Complete dose-response relationships for 5-HT and mCPBG (Figure 9b,c) as well as 1-PBG, 2-methyl 5-HT, 5HTQ, quipazine, DA and mCPP indicate a decreased nH and a higher affinity for agonists in 5-HT3-B-expressing cells (5-HT: EC50: 200 +/- 29 nM (n=8) compared to 540 +/- 50 nM (n=7), $p< 0.005$).

Dose response for 5-HT -activated Ca^{2+} influx using the FLIPR system is shown in Figure 9b. 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) had significantly higher affinity toward 5-HT and decreased nH compared to 5-HT3-A homomeric receptors (squares). Peak responses were determined and normalized to the maximum observed response. Mean values from 4-10 separate experiments are presented. Data were fit with Boltzmann exponentials.

Dose response for mCPBG-activated Ca^{2+} influx using the FLIPR system. Data from 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) and 5-HT3-A homomeric receptors (squares) is shown in figure 9c. Peak responses were determined and normalized to the maximum observed response. Values were averaged from 4 separate experiments. Data were fit with Boltzmann exponentials.

Data acquired during fast application of agonist in the FLIPR system measuring receptor-mediated Ca^{2+} influx indicate that all three agonists behaved similarly: cells co-expressing both subunits revealed a shallower dose-response relationship shifted to higher affinity. A likely reason for the difference between the 5-HT dose response in oocytes and in recombinant mammalian cells is that the fast desensitization of the 5-HT response produced an apparent shift in affinity during the relatively slow application of 5-HT in the oocyte studies. This was not observed for 1-PBG and mCPBG, presumably because the responses to these agonists desensitized more slowly (Table 1).

TABLE 1

The t80 of current responses to prolonged exposure to 5-HT3 receptors is altered in *Xenopus* oocytes expressing heteromers of both 5-HT3-A and 5-HT3-B subunits. Mean +/- SEM (n) for peak responses obtained from a holding potential of -70 mV are shown. p values were determined using the Student's *t*-test.

	Oocytes expressing:	5-HT (10 μM)	2-Me-5-HT (10 μM)	mCPBG (10 μM)	1-PBG (100 μM)
slow control 5-HT response	5-HT3-A	27.1 +/- 3/0 (38)	35 (1)	31.9 +/- 6.3 (9)	31.7 +/- 2.9 (11)
	5-HT3-A + 5-HT3-B	4.6 +/- 0.3 (33) ($p<5e^{-9}$)	7.3 +/- 0.3 (2)	15.8 +/- 1.6 (7) ($p<0.05$)	19.1 +/- 2.1 (10) ($p<0.005$)
fast control 5-HT response	5-HT3-A	1.6 +/- 0.2 (11)	2.6 +/- 1.4 (2)	3.6 +/- 1.4 (7)	4.3 +/- 1.3 (5)
	5-HT3-A + 5-HT3-B	5.6 +/- 0.6 (12) ($p<5e^{-5}$)	9.7 +.- 1.6 (5) ($p<0.05$)	22.2 +/- 3.2 (11) ($p<1e^{-4}$)	17.5 +/- 1.6 (7) ($p<e^{-4}$)

The differences in kinetics and pharmacology observed in the Ca^{2+} influx experiments were not due to altered intracellular Ca^{2+} buffering in the Ca^{2+}-influx studies. Similar results were observed for 5-HT and 1-PBG-elicited whole-cell currents (Figure 9d). In cells expressing only 5-HT3-A receptors, 10 μM 1-PBG (solid bar) produced a small inward current and increase in conductance (Figure 9d top left). Cells were challenged with a voltage ramp protocol to simultaneously determine whole cell conductance changes (1 Hz); the ramp-induced currents (spikes in Figure 9d) are shown on a faster time scale in Figure 9e. The cell subsequently revealed a large 5-HT response (clear bar indicates when 10 μM 5-HT was applied). In double transfectants, the response to 10 μM 1-PBG was larger in cells that produced comparable 5-HT responses (Figure 9d top right). Top: inward currents elicited by 10 μM 1-PBG (solid bar) and 10 μM 5-HT (clear bar) from a holding potential of -68 mV. Bottom: inward currents elicited by 100 μM 1-PBG (hatched bar). 1-PBG (10 μM) elicited responses that were 3 +/- 1 % (n=7) of the response to 10 μM 5-HT in individual 5-HT3-A/HEK and 10-fold higher (28 and 34%) of the response to 10 μM 5-HT in individual 5-HT3-A/5-HT3-B/HEK cells. The rate of decay of the response to 5-HT (10 μM; Figure 9d top, clear bar) and 1-PBG (30 μM; Figure 9d bottom, hatched bar) was usually accelerated in the presence of 5-HT3-B.

The voltage dependence of the 5-HT induced current was rectifying in 5-HT3-A-expressing cells and more linear in the presence of 5-HT3-B (Figure 9e). 5-HT induced currents from transfected HEK293 cells expressing the homomeric receptor (left) and 5-HT3-A and 5-HT3-B subunits (right). A voltage ramp was applied to the cell from -120 to +80 mV (not corrected for a junction potential of -18 mV) over 200 msec (1 mV/ms) before (solid circle) and during (clear circle) agonist application. The V_{rev} were similar for both responses and were near 0 mV. Asterisks above the recordings in (d) indicate the ramp currents shown on an expanded scale in (e). The ratio of 5-HT induced current measured 50 mV positive and negative from V_{rev} was calculated as an indication of the degree of rectification. In 5-HT3-A/HEK cells, the inward current was 2-fold larger than the outward current (ratio: 0.53 +/- 0.04; n=10).

In cells expressing both subunits, the current-voltage relationship was linear (ratio: 1.07 +/- 0.10; n=5). This rectification difference observed between the two transfectants was statistically different ($p< 0.005$).

In all cell lines tested, the Ca^{+2} influx observed during challenge with agonists at concentrations near their EC50 was completely blocked by the specific 5-HT3 receptor antagonists tropisetron (IC50 ~ 10 nM), LY 278584 (IC50 ~6 nM) and MDL 72222 (IC50 ~ 15 nM). Ketanserin, an antagonist at 5-HT_2 receptors, had no effect on 5-HT or 1-PBG induced Ca^{2+} responses up to 10 μM. Spiperone, an antagonist at 5-HT_{2A} and D_2 DA receptors, appeared to be a partial agonist at the 5-HT3-A receptor at concentrations above 1 μM.

The mechanism underlying the altered kinetics in the presence of 5-HT3-B is not known. In one possibility, 5-HT3-B allosterically modulates the rate of desensitization. If this were the case, then one prediction is a decrease in the peak currents in 5-HT3-B containing receptors compared to homomeric receptors (which was not observed), but this prediction assumes no change in single channel conductance. Heteromeric channels in fact have a significantly larger single channel conductance compared to homomeric channels (Davies et al., 1999). In a second possibility, 5-HT3-B alters the rate for agonist binding such that the first latency is shorter in heteromers compared to homomers and the rate to desensitize is not altered. A similar model was presented for the sodium channel (Aldrich et al., 1983). The time between 80% peak values on the rising and falling phases of the response (t80) appeared to be proportional to the time to peak response, which is consistent with the latter mechanism. In the majority of oocytes, the time to peak was 2-fold faster if 5-HT3-A was co-injected with 5-HT3-B. Furthermore, the altered pharmacology is consistent with a decrease in first latency for channel opening. The effect observed (a decreased nH and a decreased EC_{50} in the presence of 5-HT3-B) may be explained by a loss of negative cooperativity (Liu and Dilger, 1993) when 5-HT3-A subunits associate with 5-HT3-B subunits, which have no detectable functional binding site for agonists. In this model, binding of one agonist decreases the affinity of other agonist

binding sites for agonist in the receptor complex. 5-HT3 receptors reveal a very strong cooperativity—Hill coefficients have been determined from functional studies to be near 2 or 3 (Brown et al., 1998; Sepulveda et al., 1991; Zhong et al., 1999). In a model in which the first latency decreases while the rate of desensitization remains unchanged, there is predicted to be an increased peak current due to channel opening within a shorter time window, which was observed. Single channel analysis may be prohibitive in this system since homomeric receptors have sub-pS single channel conductances (Davies et al., 1999). Only the heteromeric receptors have a large enough single channel conductance to reliably measure single channel openings (Davies et al., 1999).

EXAMPLE 7

Binding assay on human 5-HT3-B and 5-HT3-A co-transfected mammalian cells.

HEK293 cells stably expressing 5-HT3-A receptor with or without human 5-HT3-B can be used in ^{3}H-[mCPBG] binding assays. Equilibrium ligand binding assays can be performed using conventional procedures (Lummis and Baker, 1997; Lummis et al., 1993). Specific ^{3}H-[mCPBG] binding is observed in membrane preparations from 5-HT3 receptor and human 5-HT3-B transfected cells. Oocytes expressing 5-HT3-A and human 5-HT3-B can be used to measure the affinity of binding of other compounds and their ability to displace ^{3}H-[mCPBG] binding.

EXAMPLE 8

Cloning of the human 5-HT3-B cDNA into E. coli Expression Vectors

Recombinant human 5-HT3-B is produced in E. coli following the transfer of the expression cassette into E. coli expression vectors, including but not limited to, the pET series (Novagen). The pET vectors place human 5-HT3-B expression under control of the tightly regulated bacteriophage T7 promoter. Following transfer of this construct into an E. coli host that contains a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of

human 5-HT3-B is induced when an appropriate lac substrate (IPTG) is added to the culture. The levels of expressed human 5-HT3-B are determined by the assays described herein.

The cDNA encoding the entire open reading frame for human 5-HT3-B is inserted into the NdeI site of pET [16]11a. Constructs in the positive orientation are identified by sequence analysis and used to transform the expression host strain BL21. Transformants are then used to inoculate cultures for the production of human 5-HT3-B protein. Cultures may be grown in M9 or ZB media, whose formulation is known to those skilled in the art. After growth to an OD_{600} to approximately 1.5, expression of human 5-HT3-B is induced with 1 mM IPTG for 3 hours at 37°C.

EXAMPLE 9

Cloning of human 5-HT3-B cDNA into a Baculovirus Expression Vector for Expression in Insect Cells

Baculovirus vectors, which are derived from the genome of the AcNPV virus, are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL# 1711). Recombinant baculoviruses expressing human 5-HT3-B cDNA is produced by the following standard methods (InVitrogen Maxbac Manual): the human 5-HT3-B cDNA constructs are ligated into the polyhedrin gene in a variety of baculovirus transfer vectors, including the pAC360 and the BlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA [Kitts, P.A., Nuc. Acid. Res. 18: 5667 (1990)] into Sf9 cells. Recombinant pAC360 viruses are identified by the absence of inclusion bodies in infected cells and recombinant pBlueBac viruses are identified on the basis of ß-galactosidase expression (Summers, M. D. and Smith, G. E., Texas Agriculture Exp. Station Bulletin No. 1555). Following plaque purification, human 5-HT3-B expression is measured by the assays described herein.

The cDNA encoding the entire open reading frame for human 5-HT3-B is inserted into the BamHI site of pBlueBacII. Constructs in the positive orientation are identified by sequence analysis and used to transfect Sf9 cells in the presence of linear AcNPV mild type DNA.

Authentic, active human 5-HT3-B is found in the cytoplasm of infected cells. Active human 5-HT3-B is extracted from infected cells by hypotonic or detergent lysis.

EXAMPLE 10

Cloning of human 5-HT3-B cDNA into a yeast expression vector

Recombinant human 5-HT3-B is produced in the yeast S. cerevisiae following the insertion of the optimal human 5-HT3-B cDNA cistron into expression vectors designed to direct the intracellular or extracellular expression of heterologous proteins. In the case of intracellular expression, vectors such as EmBLyex4 or the like are ligated to the human 5-HT3-B cistron [Rinas, U. et al., Biotechnology 8: 543-545 (1990); Horowitz B. et al., J. Biol. Chem. 265: 4189-4192 (1989)]. For extracellular expression, the human 5-HT3-B cistron is ligated into yeast expression vectors which fuse a secretion signal (a yeast or mammalian peptide) to the NH_2 terminus of the human 5-HT3-B protein [Jacobson, M. A., Gene 85: 511-516 (1989); Riett L. and Bellon N. Biochem. 28: 2941-2949 (1989)].

These vectors include, but are not limited to pAVE1>6, which fuses the human serum albumin signal to the expressed cDNA [Steep O. Biotechnology 8: 42-46 (1990)], and the vector pL8PL which fuses the human lysozyme signal to the expressed cDNA [Yamamoto, Y., Biochem. 28: 2728-2732)]. In addition, human 5-HT3-B is expressed in yeast as a fusion protein conjugated to ubiquitin utilizing the vector pVEP [Ecker, D. J., J. Biol. Chem. 264: 7715-7719 (1989), Sabin, E. A., Biotechnology 7: 705-709 (1989), McDonnell D. P., Mol. Cell Biol. 9: 5517-5523 (1989)]. The levels of expressed human 5-HT3-B are determined by the assays described herein.

EXAMPLE 11

Purification of Recombinant human 5-HT3-B

Recombinantly produced human 5-HT3-B may be purified by antibody affinity chromatography.

Human 5-HT3-B antibody affinity columns are made by adding the anti-human 5-HT3-B antibodies to Affigel-10 (Bio-Rad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents and the cell culture supernatants or cell extracts containing solubilized human 5-HT3-B are slowly passed through the column. The column is then washed with phosphate- buffered saline together with detergents until the optical density (A280) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) together with detergents. The purified human 5-HT3-B protein is then dialyzed against phosphate buffered saline.

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WHAT IS CLAIMED IS:

1. An isolated and purified DNA molecule which encodes human 5-HT3-B protein, or a functional derivative thereof, wherein said protein functions as a human serotonin receptor subunit.

2. The isolated and purified DNA molecule of claim 1, having a nucleotide sequence selected from a group consisting of: **(SEQ.ID.NO.:1)**; **(SEQ.ID.NO.:2)**; and functional derivatives thereof.

3. The isolated and purified DNA molecule of claim 1, wherein said DNA molecule is genomic DNA.

4. An expression vector for expression of human 5-HT3-B protein in a recombinant host, wherein said vector contains a recombinant gene encoding human 5-HT3-B protein according to claim 1, or a functional derivative thereof.

5. The expression vector of claim 4, wherein the expression vector contains a cloned gene encoding human 5-HT3-B protein wherein said protein functions as a human serotonin receptor subunit, having a nucleotide sequence selected from a group consisting of: (SEQ.ID.NO.:1); (SEQ.ID.NO.:2); and functional derivatives thereof.

6. The expression vector of claim 4, wherein the expression vector contains genomic DNA encoding human 5-HT3-B protein.

7. A recombinant host cell containing a recombinantly cloned gene encoding human 5-HT3-B protein wherein said protein functions as a human serotonin receptor subunit, or functional derivative thereof.

8. The recombinant host cell of claim 7, wherein said gene has a nucleotide sequence selected from a group consisting of: (SEQ.ID.NO.:1); (SEQ.ID.NO.:2); and functional derivatives thereof.

9. The recombinant host cell of claim 7, wherein said cloned gene encoding human 5-HT3-B protein is genomic DNA.

10. A protein, in substantially pure form which functions as human 5-HT3-B protein and wherein said protein functions as a modifier of the human 5-HT3-A receptor.

11. The protein according to claim 10, having an amino acid sequence selected from a group consisting of: (SEQ.ID.NO.:3); (SEQ.ID.NO.:4); and functional derivatives thereof.

12. A monospecific antibody immunologically reactive with human 5-HT3-B protein wherein said protein functions as a modifier of the human 5-HT3-A receptor.

13. The antibody of Claim 12, wherein the antibody blocks activity of the 5-HT3-B subunit of the human serotonin receptor.

14. A process for expression of human 5-HT3-B protein in a recombinant host cell, comprising:

(a) transferring the expression vector of Claim 4 into suitable host cells; and

(b) culturing the host cells of step (a) under conditions which allow expression of the human 5-HT3-B protein from the expression vector.

15. A method of identifying compounds that modulate human 5-HT3-B protein activity, comprising:

(a) combining a modulator of human 5-HT3-B protein activity with human 5-HT3-B protein wherein said protein optionally functions as a modifier of the human 5-HT3-A receptor; and

(b) measuring an effect of the modulator on the protein.

16. The method of claim 15, wherein the effect of the modulator on the protein is inhibiting or enhancing binding of human 5-HT3 receptor ligands.

17. The method of claim 15, wherein the effect of the modulator on the protein is stimulation or inhibition of human 5-HT3-B-containing serotonin receptor.

18. The method of claim 17, wherein the human 5-HT3-B is altering the kinetics and the pharmacology of the human 5-HT3-B-containing serotonin receptor.

19. A compound active in the method of Claim 15, wherein said compound is a modulator of a human 5-HT3-B containing serotonin receptor.

20. A compound active in the method of Claim 15, wherein said compound is an agonist or antagonist of a subclass of 5-HT3 receptor consisting of both 5-HT3-A and 5HT3B proteins.

21. A compound active in the method of Claim 15, wherein said compound is a modulator of expression of a 5-HT3-B subunit.

22. A pharmaceutical composition comprising a compound active in the method of Claim 15, wherein said compound is a modulator of human 5-HT3-B subunit activity.

23. A method of treating a patient in need of such treatment for a condition which is mediated by a human 5-HT3-B-containing serotonin receptor , comprising administration of a human 5-HT3-B modulating compound active in the method of Claim 15.

5

TITLE OF THE INVENTION

DNA ENCODING A HUMAN SUBUNIT OF THE 5-HT3 SEROTONIN RECEPTOR

5

ABSTRACT OF THE INVENTION

DNA encoding human 5-HT3-B has been cloned and characterized. The recombinant protein is capable of forming biologically active human 5-HT3-B protein. The cDNA has been expressed in recombinant host cells that produce active
10 recombinant protein. In addition, the recombinant host cells are utilized to establish a method for identifying modulators of the receptor activity, and receptor modulators are identified.

FIGURE 1

[SEQ.ID.NO.:5] Nucleic Acid sequence of the human 5-HT3-B (full sequence including untranslated regions); 1923 bases. (141 bases of 5' UTR; 456 bases of 3' UTR)

CCACGCGTCCGTAAGGATAGCATCAACTGGCAAACGGAGAAGGAGGAGAA
CAGAGTGGAGAGGAACCCTGTTAGGAGAAATTGAGCGGCATTCCATCTGG
TAGGCAAGTTTGCATTTCTCCTTTTTGGGATCTGCCCAGGA**ATG**TTGTCA
AGTGTAATGGCTCCCCTGTGGGCCTGCATCCTGGTGGCTGCAGGAATTCT
AGCCACAGATACACATCATCCCCAGGATTCTGCTCTGTATCATCTCAGCA
AGCAGCTATTACAGAAATATCATAAAGAAGTGAGACCTGTTTACAACTGG
ACCAAGGCCACCACAGTCTACCTGGACCTGTTCGTCCATGCTATATTGGA
TGTGGATGCAGAGAATCAAATATTAAAGACAAGTGTATGGTACCAAGAGG
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TGAACTCATCTGGGACCATTGAGAACTATAAGCCCATCCAGGTGGTCTCT
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CCTGACCTTCAAGAGCATTCTGCATACAGTGGAAGACGTAGACCTGGCCT
TTCTGAGGAGCCCAGAAGACATTCAGCATGACAAAAAGGCGTTTTTGAAT
GACAGTGAGTGGGAACTTCTATCTGTGTCCTCCACATACAGCATCCTGCA
GAGCAGCGCTGGAGGATTTGCACAGATTCAGTTTAATGTGGTGATGCGCA
GGCACCCCCTGGTCTATGTCGTGAGTCTGCTGATTCCTAGCATCTTTCTC
ATGCTGGTGGACCTGGGGAGCTTCTACCTGCCACCCAACTGCCGAGCCAG
GATTGTGTTCAAGACCAGTGTGCTGGTGGGCTACACCGTCTTCAGGGTCA
ACATGTCCAACCAGGTGCCACGGAGTGTAGGGAGCACCCCTCTGATTGGG
CACTTCTTCACCATCTGCATGGCCTTCTTGGTTCTCAGCTTAGCTAAGTC
CATCGTGTTGGTCAAATTCCTCCATGATGAGCAGCGTGGTGGACAGGAGC
AGCCCTTCTTGTGCCTTCGAGGGGACACCGATGCTGACAGGCCTAGAGTG
GAACCCAGGGCCCAACGTGCTGTGGTAACAGAGTCCTCGCTGTATGGAGA
GCACCTGGCCCAGCCAGGAACCCTGAAGGAAGTCTGGTCGCAGCTTCAAT
CTATCAGCAACTACCTCCAAACTCAGGACCAGACAGACCAACAGGAGGCA
GAGTGGCTGGTCCTCCTGTCCCGCTTTGACCGACTGCTCTTCCAAAGCTA
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TGTGGGGCGGCGTG**TGA**AGACTGAAGTGTTCTTCAGTAATTGTGCTGGCA
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CGGGTGTGGTGGTTCTTGCCTATAGTCCCAGTGCTTTGGGAGGCCATAGC
AGGAGGATTGCTTGAGCCCAGGAGTTCGAGACCAGCCAGAGCAACATAGT
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ATAAATAAATAGCTGGGCATAGTGGCTCATGCCTGTACTCTCAGCTACTT
GGGAGGTTGAGGTGGGAGGATTGCTTGAGCCCAGGATTTCAAGGCTGCAG
TGAGCCATGATTGCACCACTGCACCCCAGCCTGGGTGACAGAACAAGACC
CTGTCTCAAAAAAAAAAATAAAATAAAAGGCTTTCTGCCTTCAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAG

FIGURE 2

[SEQ.ID.NO.:6] Nucleotide sequence for the coding region of 5-HT3-B is shown; 1326 bases.

ATGTTGTCAAGTGTAATGGCTCCCCTGTGGGCCTGCATCCTGGTGGCTGC
AGGAATTCTAGCCACAGATACACATCATCCCCAGGATTCTGCTCTGTATC
ATCTCAGCAAGCAGCTATTACAGAAATATCATAAAGAAGTGAGACCTGTT
TACAACTGGACCAAGGCCACCACAGTCTACCTGGACCTGTTCGTCCATGC
TATATTGGATGTGGATGCAGAGAATCAAATATTAAAGACAAGTGTATGGT
ACCAAGAGGTCTGGAATGATGAATTTTTATCCTGGAACTCCAGCATGTTT
GATGAGATTAGAGAGATCTCCCTACCTCTAAGTGCCATCTGGGCCCCCGA
TATCATCATCAATGAGTTTGTGGACATTGAAAGATACCCTGACCTTCCCT
ATGTTTATGTGAACTCATCTGGGACCATTGAGAACTATAAGCCCATCCAG
GTGGTCTCTGCGTGCAGTTTAGAGACATATGCTTTTCCATTTGATGTCCA
GAATTGCAGCCTGACCTTCAAGAGCATTCTGCATACAGTGGAAGACGTAG
ACCTGGCCTTTCTGAGGAGCCCAGAAGACATTCAGCATGACAAAAAGGCG
TTTTTGAATGACAGTGAGTGGGAACTTCTATCTGTGTCCTCCACATACAG
CATCCTGCAGAGCAGCGCTGGAGGATTTGCACAGATTCAGTTTAATGTGG
TGATGCGCAGGCACCCCCTGGTCTATGTCGTGAGTCTGCTGATTCCTAGC
ATCTTTCTCATGCTGGTGGACCTGGGGAGCTTCTACCTGCCACCCAACTG
CCGAGCCAGGATTGTGTTCAAGACCAGTGTGCTGGTGGGCTACACCGTCT
TCAGGGTCAACATGTCCAACCAGGTGCCACGGAGTGTAGGGAGCACCCCT
CTGATTGGGCACTTCTTCACCATCTGCATGGCCTTCTTGGTTCTCAGCTT
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GACAGGAGCAGCCCTTCTTGTGCCTTCGAGGGGACACCGATGCTGACAGG
CCTAGAGTGGAACCCAGGGCCCAACGTGCTGTGGTAACAGAGTCCTCGCT
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AGCTTCAATCTATCAGCAACTACCTCCAAACTCAGGACCAGACAGACCAA
CAGGAGGCAGAGTGGCTGGTCCTCCTGTCCCGCTTTGACCGACTGCTCTT
CCAAAGCTACCTTTTCATGCTGGGGATCTACACCATCACTCTGTGCTCCC
TCTGGGCACTGTGGGGCGGCGTGTGA

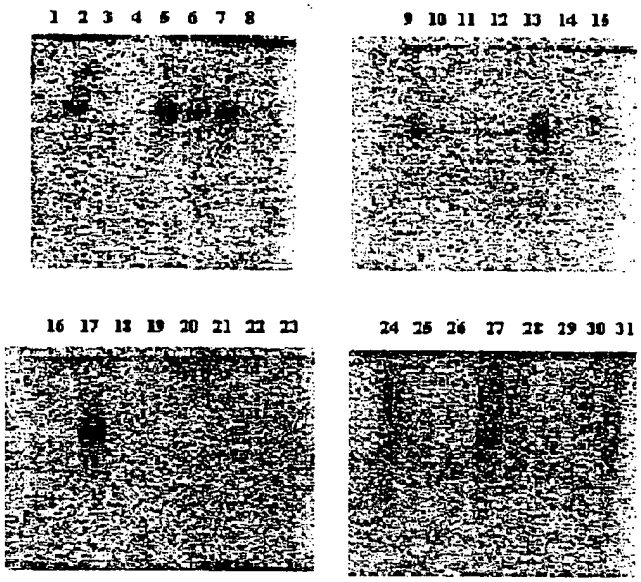
FIGURE 3

[SEQ.ID.NO.:7] The amino acid sequence of 5-HT3-B is shown (441 amino acids).

Met Leu Ser Ser Val Met Ala Pro Leu Trp Ala Cys Ile Leu Val Ala Ala Gly Ile
Leu Ala Thr Asp Thr His His Pro Gln Asp Ser Ala Leu Tyr His Leu Ser
Lys Gln Leu Leu Gln Lys Tyr His Lys Glu Val Arg Pro Val Tyr Asn Trp Thr
Lys Ala Thr Thr Val Tyr Leu Asp Leu Phe Val His Ala Ile Leu Asp Val Asp
Ala Glu Asn Gln Ile Leu Lys Thr Ser Val Trp Tyr Gln Glu Val Trp Asn Asp
Glu Phe Leu Ser Trp Asn Ser Ser Met Phe Asp Glu Ile Arg Glu Ile Ser Leu
Pro Leu Ser Ala Ile Trp Ala Pro Asp Ile Ile Ile Asn Glu Phe Val Asp Ile
Glu Arg Tyr Pro Asp Leu Pro Tyr Val Tyr Val Asn Ser Ser Gly Thr Ile Glu
Asn Tyr Lys Pro Ile Gln Val Val Ser Ala Cys Ser Leu Glu Thr Tyr Ala Phe
Pro Phe Asp Val Gln Asn Cys Ser Leu Thr Phe Lys Ser Ile Leu His Thr Val
Glu Asp Val Asp Leu Ala Phe Leu Arg Ser Pro Glu Asp Ile Gln His Asp Lys
Lys Ala Phe Leu Asn Asp Ser Glu Trp Glu Leu Leu Ser Val Ser Ser Thr Tyr
Ser Ile Leu Gln Ser Ser Ala Gly Gly Phe Ala Gln Ile Gln Phe Asn Val Val
Met Arg Arg His Pro Leu Val Tyr Val Val Ser Leu Leu Ile Pro Ser Ile Phe
Leu Met Leu Val Asp Leu Gly Ser Phe Tyr Leu Pro Pro Asn Cys Arg Ala Arg
Ile Val Phe Lys Thr Ser Val Leu Val Gly Tyr Thr Val Phe Arg Val Asn Met
Ser Asn Gln Val Pro Arg Ser Val Gly Ser Thr Pro Leu Ile Gly His Phe Phe
Thr Ile Cys Met Ala Phe Leu Val Leu Ser Leu Ala Lys Ser Ile Val Leu Val
Lys Phe Leu His Asp Glu Gln Arg Gly Gly Gln Glu Gln Pro Phe Leu Cys Leu
Arg Gly Asp Thr Asp Ala Asp Arg Pro Arg Val Glu Pro Arg Ala Gln Arg Ala
Val Val Thr Glu Ser Ser Leu Tyr Gly Glu His Leu Ala Gln Pro Gly Thr Leu
Lys Glu Val Trp Ser Gln Leu Gln Ser Ile Ser Asn Tyr Leu Gln Thr Gln Asp
Gln Thr Asp Gln Gln Glu Ala Glu Trp Leu Val Leu Leu Ser Arg Phe Asp Arg
Leu Leu Phe Gln Ser Tyr Leu Phe Met Leu Gly Ile Tyr Thr Ile Thr Leu Cys
Ser Leu Trp Ala Leu Trp Gly Gly Val

FIGURE 4 PANEL A

The tissue distribution of 5-HT3-B is shown.



1, Cerebellum; 2, Cerebral cortex; 3, Medulla; 4, Spinal Cord; 5, Occipital pole; 6, frontal lobe; 7, Temporal lobe; 8, Putamen; 9, Amygdala; 10, Caudate nucleus; 11, Corpus callosum; 12, Hippocampus; 13, Whole brain; 14, Substantia nigra; 15, Thalamus; 16, Heart; 17, Brain; 18, Placenta; 19, Lung; 20, Liver; 21, Skeletal muscle; 22, Kidney; 23, Pancreas; 24, Spleen; 25, Thymus; 26, Prostate; 27, Testis; 28, Ovary; 29, Small Intestine; 30, Colon (mucosal lining); 31, Peripheral blood leukocyte.

FIGURE 4 PANEL B

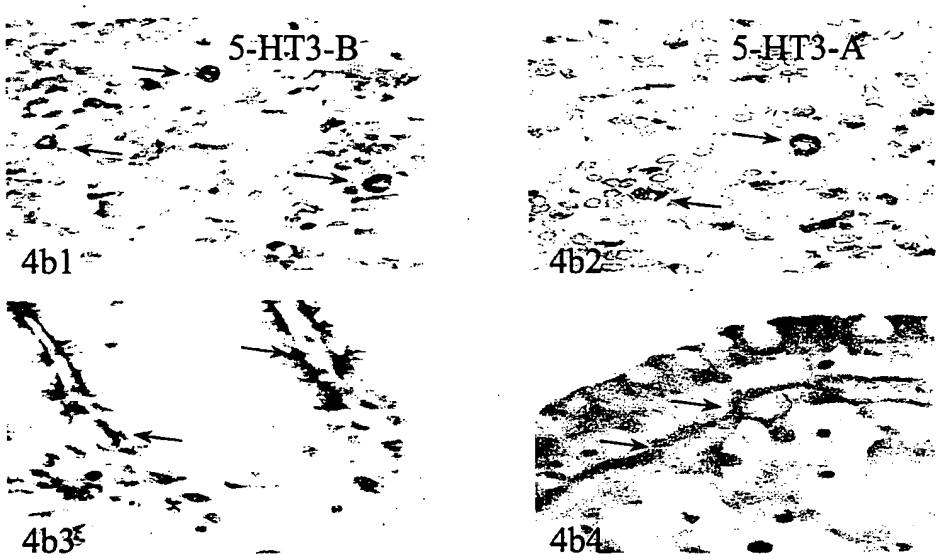


FIGURE 4 PANEL C

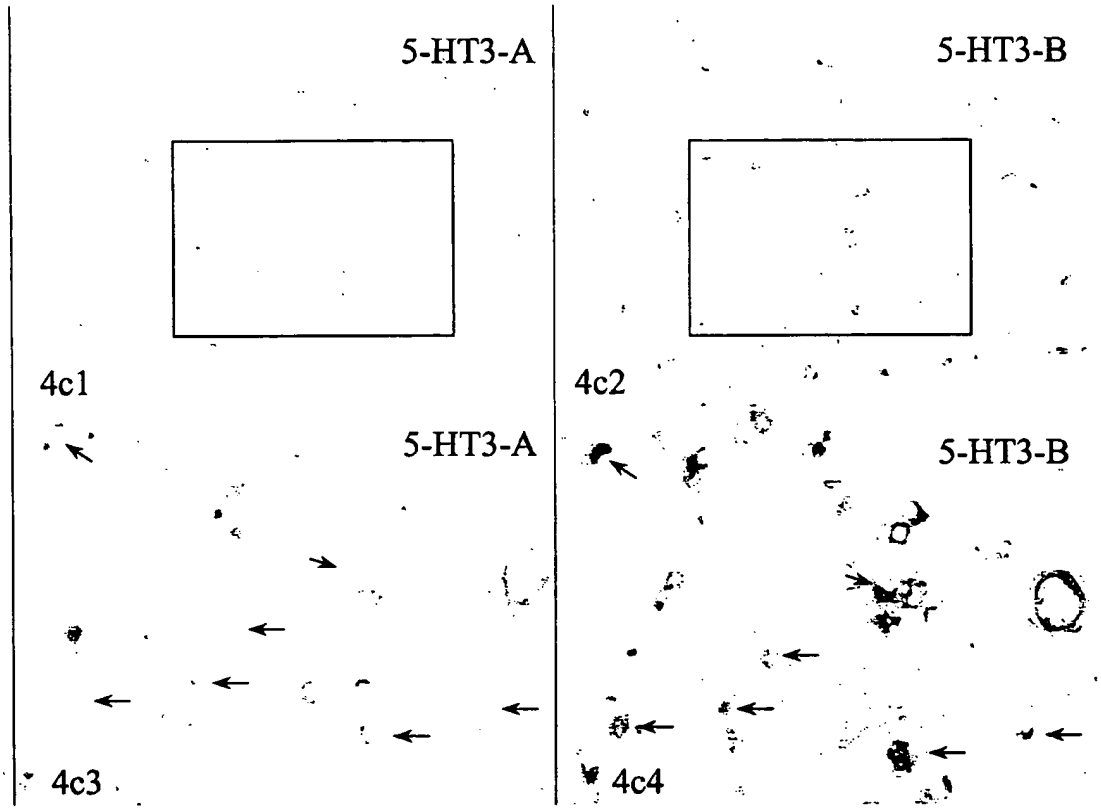


FIGURE 5 PANEL A

Figure 5

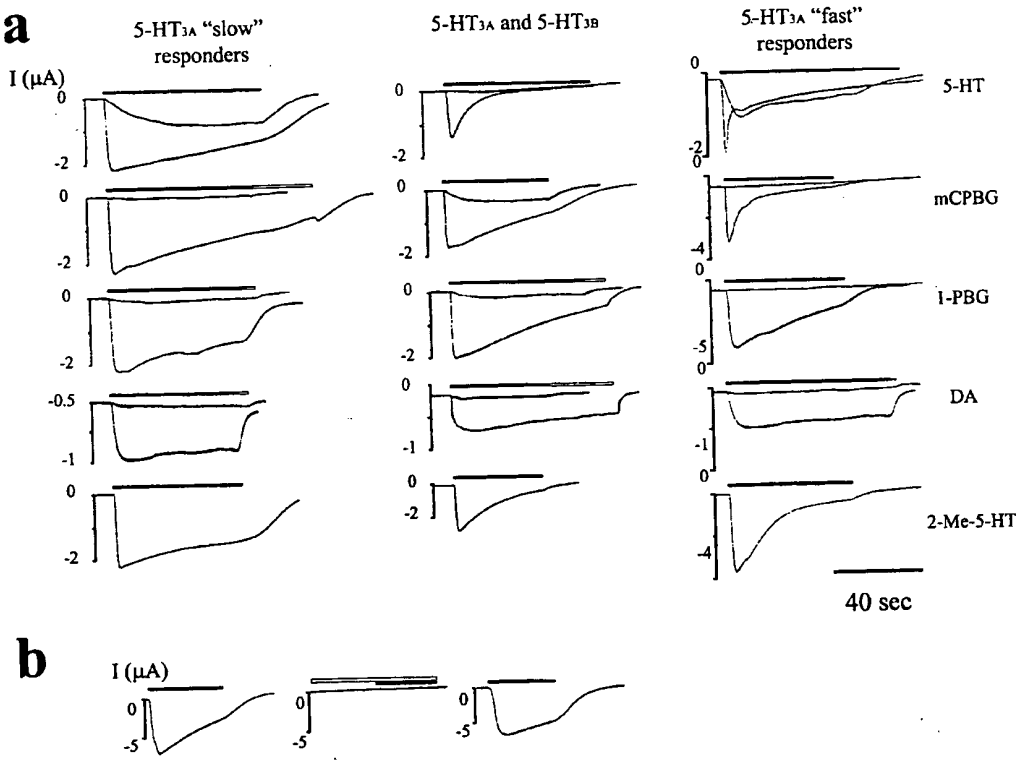


FIGURE 5 PANEL B

Figure 5

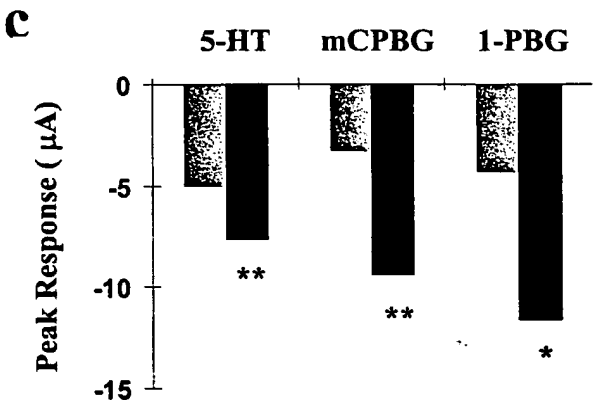


FIGURE 6

Figure 6

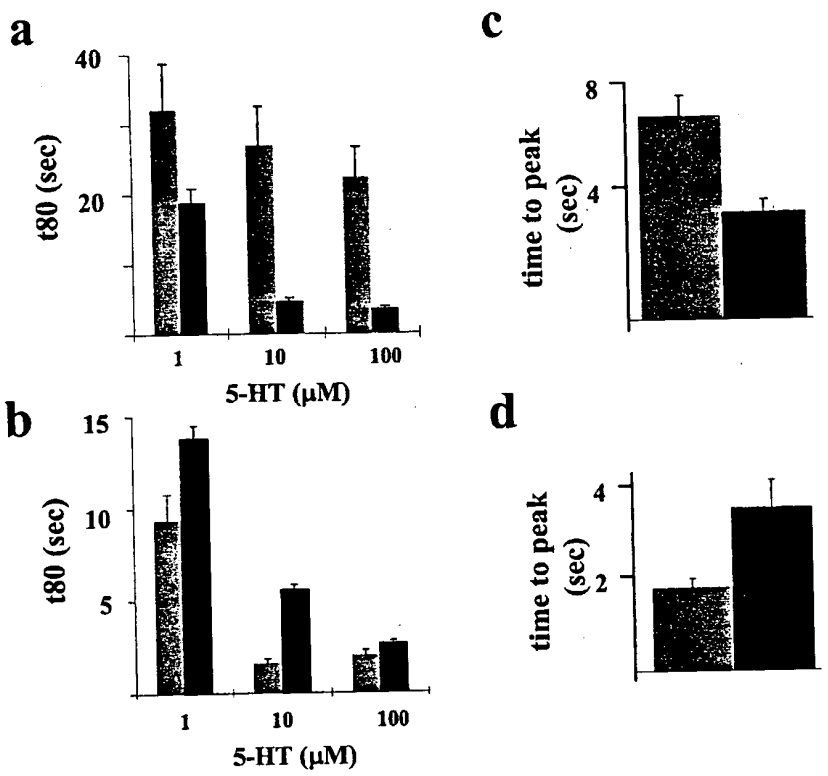


FIGURE 7

Figure 7

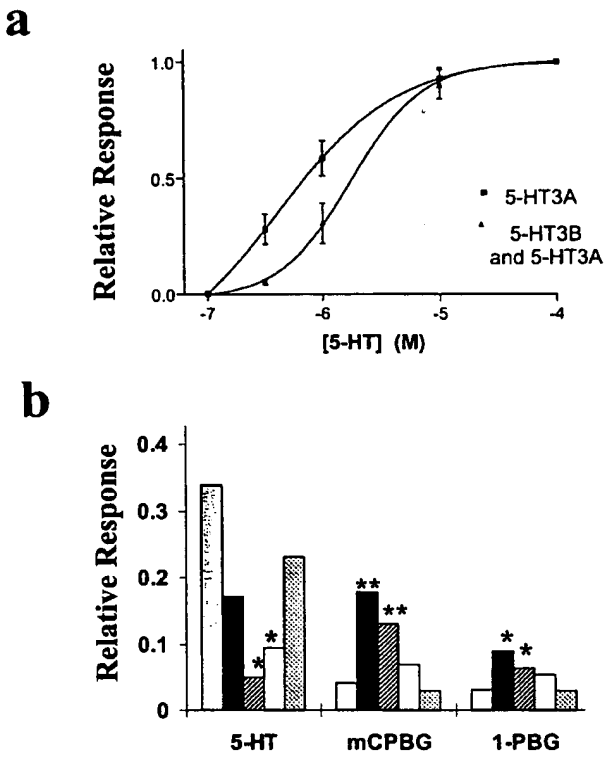


FIGURE 8

Figure 8

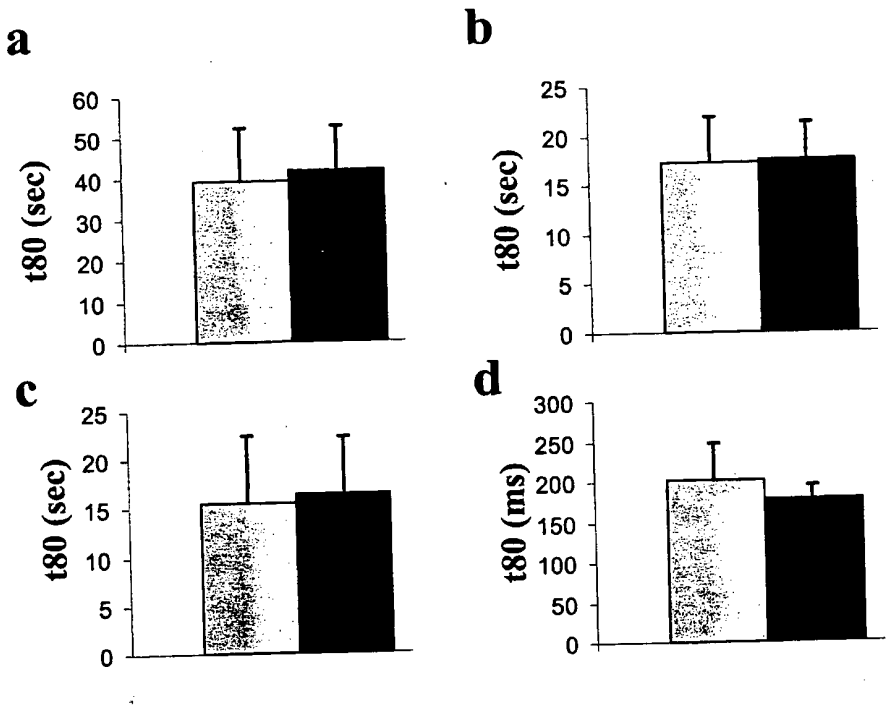
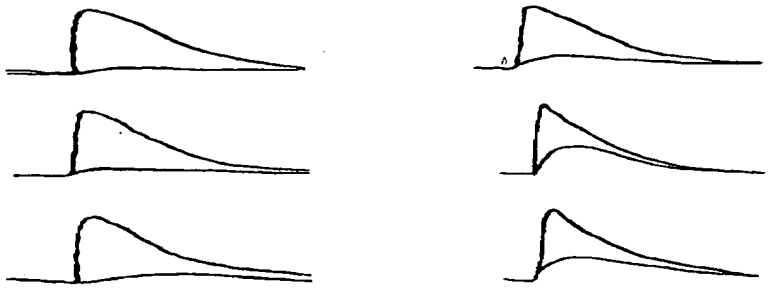


FIGURE 9 PANEL A

a



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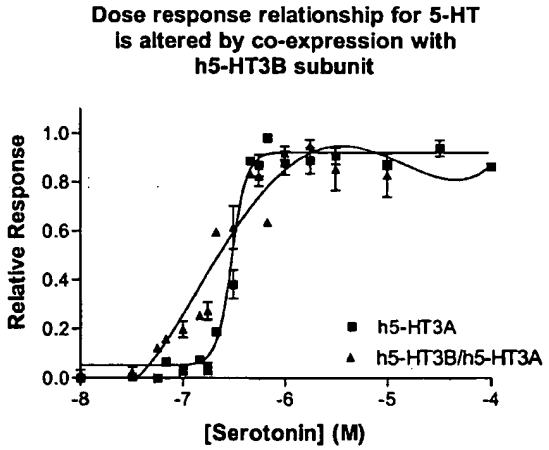


FIGURE 9 PANEL B

C

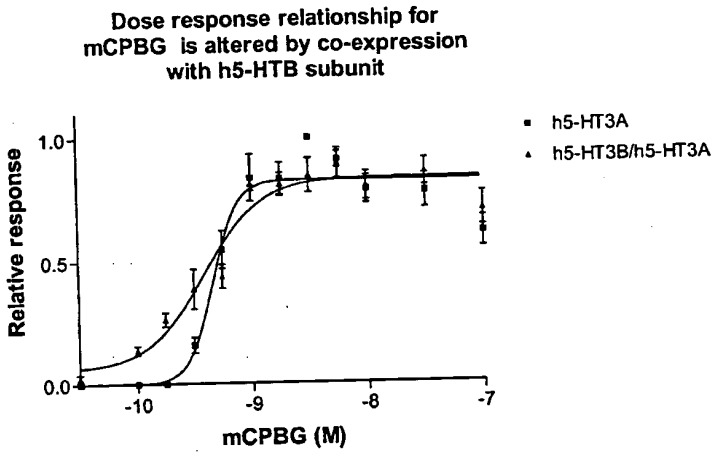
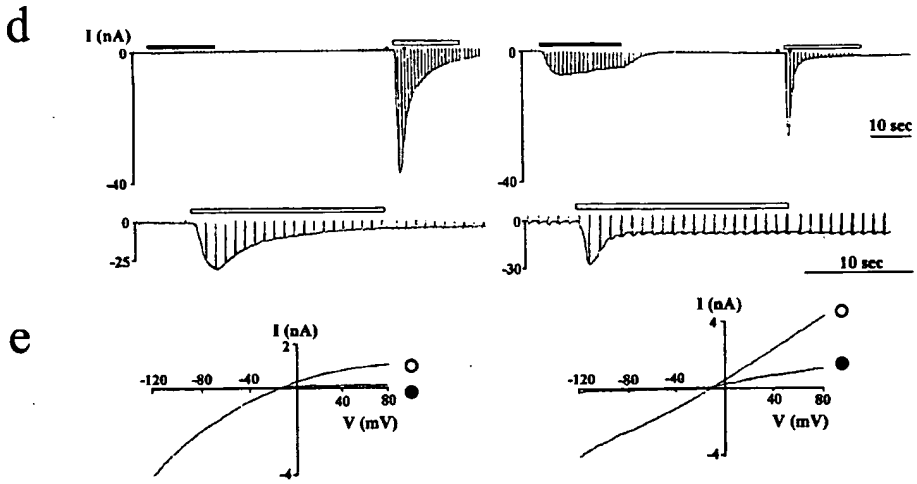


FIGURE 9 PANEL C

Figure 9



SEQUENCE LISTING

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D'Andrea, Mike
Pyati, Jaushree
Zhu, Jessica Y
Erlander, Mark G

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WO 00/71741 A3

(54) Title: DNA ENCODING A HUMAN SUBUNIT OF THE 5-HT3 SEROTONIN RECEPTOR

(57) Abstract: DNA encoding human 5-HT3-B has been cloned and characterized. The recombinant protein is capable of forming biologically active human 5-HT3-B protein. The cDNA has been expressed in recombinant host cells that produce active recombinant protein. In addition, the recombinant host cells are utilized to establish a method for identifying modulators of the receptor activity, and receptor modulators are identified.

TITLE OF THE INVENTION

DNA ENCODING A HUMAN SUBUNIT OF THE 5-HT3 SEROTONIN RECEPTOR

BACKGROUND OF THE INVENTION

Serotonin (5-hydroxytryptamine, 5-HT) is a multifunctional chemical transmitter that signals though cell surface receptors. At least fourteen subtypes of serotonin receptors have been defined pharmacologically (Julius, 1996; Tecott and Julius, 1993). Thirteen of the fourteen known receptors are G-protein coupled receptors and the only known ionotropic 5-HT receptor, the type 3 5-HT3 receptor, is a fast activating, ligand gated non-selective cation channel unique among known monoamine receptors (Derkach et al., 1989). The 5-HT3 receptor is exclusively localized on neurons in the central (Waeber et al., 1989; Yakel et al., 1991) and peripheral (Fozard, 1984) nervous systems. Activation of the 5-HT3 receptor leads to membrane depolarization and an increase in intracellular Ca^{2+}. The 5-HT3 receptor is the target of antagonists (granisetron and ondansetron) selective against the nausea induced by cytotoxic chemotherapy and general anesthesia (Gralla, 1998). Evidence is accumulating that serotonin 5-HT3 receptors are important in pain reception, anxiety, cognition, cranial motor neuron activity, sensory processing, modulation of affect, and the behavioral consequences of drug abuse (Lambert et al., 1995; Passani and Corradetti, 1996).

The 5-HT3 receptor is thought to be a homopentimeric protein with multiple agonist and allosteric ligand binding sites (Boess et al., 1995; Bonhaus et al., 1995; Eglen and Bonhaus, 1996; Green et al., 1995; Hargreaves et al., 1996; Lambert et al., 1995; Van Hooft et al., 1997; Wetzel et al., 1998). The full coding sequence of the 5-HT3 receptor has been cloned from mouse (Hope et al., 1993; Maricq et al., 1991; Werner et al., 1994), rat (Miyake et al., 1995), guinea pig (Lankiewicz et al., 1998), and human (Belelli et al., 1995; Miyake et al., 1995); AJ003079 Bruss et al., unpublished). It has structural and functional similarities with nicotinic, GABA-

ergic and other ligand gated ion channels (Barnard, 1996; Gurley and Lanthorn, 1998; Maricq et al., 1991). Like other receptors of the same ligand-gated cation channel superfamily (Changeux and Edelstein, 1998; Lena and Changeux, 1993), the 5-HT3 receptors rapidly desensitize (Peters and Lambert, 1989; Yakel et al., 1991). The pharmacological and kinetic profile of the members of this superfamily depends on subunit composition (Chang et al., 1995; Harris et al., 1995; Lindstrom et al., 1990; Luetje and Patrick, 1991; Olsen, 1998).

While it is thought that only one gene encoding the 5-HT3 receptor exists (5-HT3-A receptor), several lines of evidence indicate that 5-HT3 receptors may exist as heteromultimers. First, receptors purified from a variety of sources by affinity chromatography usually reveal at least 2 major protein bands with molecular masses in the order of 54 and 38 kDa (Lambert et al., 1995). The 5-HT3-A receptor corresponds to the former (Turton et al., 1993). Affinity purified 5-HT3 receptor solubilized from pig cerebral cortex is composed of at least 3 separable components, based on silver staining of proteins on denaturing gels (Fletcher and Barnes, 1997). A number of these protein bands are not recognized by specific antibodies directed against the recombinant 5-HT3-A subunit (Fletcher and Barnes, 1997), and their sizes are too large (52-71 kDa) to be considered as degraded 5-HT3-A fragments (Fletcher and Barnes, 1998).

Second, expression of the recombinant receptor in Xenopus oocytes or mammalian cell lines often do not reveal all the electrophysiological and pharmacological properties of the native receptor (Gill et al., 1995; Lambert et al., 1995; Van Hooft et al., 1997). Differences in desensitization kinetics, single channel conductance and agonist efficacy have been observed and may be due to the lack of an endogenous subunit not present in the recombinant cell lines or oocyte system. Two forms of the receptor subunit with about 98% identity have been observed in mouse, rat, guinea pig and human. The two forms differ by the insertion of 6 to 32 consecutive amino acids and may be produced by alternative splicing of a single gene

(Uetz et al., 1994; Werner et al., 1994). While most of the pharmacological and electrophysiological characteristics of the recombinant mouse isoforms are similar (Downie et al., 1994; Niemeyer and Lummis, 1998; Werner et al., 1994), the efficacy of 2-methyl-5-HT and/or m-chlorophenylbiguanide (mCPBG) differ (Downie et al., 1994; Niemeyer and Lummis, 1998; Van Hooft et al., 1997). The two variants may coexist in the same cell lines and heteromultimers containing both forms may explain some but not all of the differences observed between homomultimers of recombinant subunits and the native cells.

Third, 5-HT-3 receptors in different preparations from the same species reveal variation in electrophysiological and pharmacological properties (Fletcher and Barnes, 1998; Richardson and Engel, 1986). In particular, there appears to be heterogeneity of desensitization kinetics across cells, and even within the same cell type (Lambert et al., 1995). Differences in desensitization kinetics have been observed in NG108-15 cells under various differentiation states (Shao et al., 1991). While this difference may be due to heterogeneity of subunits, it may also be a consequence of different post-translational states of the receptor. For instance, the rate of desensitization of nicotinic acetylcholine and $GABA_A$ receptors is enhanced by phosphorylation (Raymond, 1998; Raymond et al., 1993; Swope et al., 1992). A wide range of single channel conductance values has been reported for the 5-HT3 receptor (Fletcher and Barnes, 1998), however, this difference may be due to the phosphorylation state of the receptor in different cells. Van Hooft and colleagues have shown that phosphorylation controls the conductance of 5-HT-3 receptors in N1E-115 cells (Van Hooft and Vijverberg, 1995). Furthermore, significant differences in rectification properties of the channel have been reported in different cell types (Hussy et al, 1994).

Fourth, differentiated murine N1E-115 cells express native 5-HT-3 receptors with an efficacy for 2-methyl-5-HT higher than that observed for either murine

recombinant 5-HT3-A isoform expressed in oocytes separately or together. However, co-expression of recombinant 5-HT3-A receptor in oocytes with mRNA isolated from differentiated murine N1E-115 cells reconstitutes the functional properties of the native receptor expressed on differentiated cells (Van Hooft et al., 1997).

While it has been reported that the 5-HT3 receptor can co-assemble with the nicotinic alpha4 subunit in Xenopus oocyte expression studies (Van Hooft et al., 1998), nicotinic ACh receptor subunits were not associated with native porcine brain 5-HT3 receptors (Fletcher et al., 1998).

Fifth, allosteric modulation of the receptor by Zn^{2+} has different effects on the recombinant murine 5-HT3-A receptor expressed in oocytes (enhancement) compared to its effects on 5-HT receptor-mediated currents in NCB20 cells (block; (Lovinger, 1991), the cell line from which the 5-HT3-A receptor used in these studies was cloned. The sensitivity of another member of this superfamily of ligand-gated receptors, $GABA_A$ receptors, to blockade by zinc ions is known to depend on subunit composition (Smart et al., 1994).

Recently the sequence of human 5-HT3-B was disclosed and shown to alter functional characteristics of 5-HT3-A (Davies et al 1999). The functional characteristics described for human 5-HT3-B by Davies et al. (1999) included a decreased affinity for 5-HT with no effect on the affinity for other agonists including mCPBG. This art did not find that the affinity and cooperativity for 5-HT3 receptor function were increased and decreased, respectively, by 5-HT3-B, nor did it report any affects on current kinetics.

The isolation and functional characterization of a human cDNA encoding a modifier subunit for the serotonin 5-HT3 receptor explains observations that 5-HT3 receptors from a variety of preparations have distinct pharmacological,

kinetic and voltage-dependent properties (Peters et al., 1992). Expression of this human 5-HT3 subunit, termed 5-HT3-B will further aid in discovery of serotonin function and can be used to screen for compounds that modulate function of a heteromeric receptor complex to which the 5-HT3-B receptor participates.

SUMMARY OF THE INVENTION

A DNA molecule encoding a human subunit with homology to 5-HT3-A serotonin receptor that, when co-expressed with the short form of the serotonin 5-HT3-A receptor, modifies the functional and pharmacological characteristics of the 5-HT3 receptor has been cloned and characterized. Using a recombinant expression system, functional DNA molecules encoding the human serotonin 5-HT3 receptor modifier protein (heretofore designated 5-HT3-B) have been isolated. The biological and structural properties of these proteins are disclosed, as is the amino acid and nucleotide sequence. The recombinant protein is useful to identify modulators of human 5-HT3 receptors composed of both 5-HT3 receptors and the modifier subunit (5-HT3-B). Modulators identified in the assay disclosed herein are useful as therapeutic agents, include, but are not limited to, nausea, depression, anxiety, psychoses (for example schizophrenia), urinary continence, Huntington's chorea, tardive dyskinesia, Parkinson's disease, obesity, hypertension, migraine, Gilles de la Tourette's syndrome, sexual dysfunction, drug addiction, drug abuse, cognitive disorders, learning, Alzheimer's disease, cerebral coma, senile dementia, obsessive-compulsive behavior, panic attacks, pain, social phobias, eating disorders and anorexia, cardiovascular and cerebrovascular disorders, non-insulin dependent diabetes mellitus, hyperglycemia, constipation, arrhythmia, disorders of the neuroendocrine system, stress, and spasticity, as well as acid secretin, ulcers, airway constriction, asthma, allergy, inflammation, and prostate dysfunction, and diagnostic agents. The recombinant DNA molecules and portions thereof, are useful for isolating homologues of the DNA molecules, identifying and isolating genomic equivalents of the DNA molecules, and identifying, detecting or isolating mutant forms of the DNA molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 – [SEQ.ID.NO.:5] Nucleic Acid sequence of the human 5-HT3-B (full sequence including untranslated regions) is shown;1923 bases (141 bases of 5' UTR; 456 bases of 3' UTR).

Figure 2 - [SEQ.ID.NO.:6] Nucleotide sequence for the coding region of 5-HT3-B is shown; 1326 bases.

Figure 3 - [SEQ.ID.NO.:7] The amino acid sequence of human 5-HT3-B is shown (441 amino acids).

Figure 4 – PANEL A [Figure 4A]. The PCR-based tissue distribution of the human 5-HT3-B is shown. Lanes are labeled as indicated: 1, Cerebellum; 2, Cerebral cortex; 3, Medulla; 4, Spinal Cord; 5, Occipital pole; 6, frontal lobe; 7, Temporal lobe; 8, Putamen; 9, Amygdala; 10, Caudate nucleus; 11, Corpus callosum; 12, Hippocampus; 13, Whole brain; 14, Substantia nigra; 15, Thalamus; 16, Heart; 17, Brain; 18, Placenta; 19, Lung; 20, Liver; 21, Skeletal muscle; 22, Kidney; 23, Pancreas; 24, Spleen; 25, Thymus; 26, Prostate; 27, Testis; 28, Ovary; 29, Small Intestine; 30, Colon (mucosal lining); 31, Peripheral blood leukocyte.

PANEL B [Figure 4B]. RT-PCR in situ hybridization was performed on normal human tissues of the spleen (b1, b2) and small intestine (b3, b4). Human spleen had intense 5-HT3-B mRNA (b1) and 5HT3-A mRNA (b2) signal (purple in color – dark grey in black-and-white), respectively, in small lymphocytes (arrows). Similarly, intense 5-HT3-B mRNA (b3) and 5HT3-A mRNA (b4) signal (purple in color – dark grey in black-and-white) was detected in stromal fibroblasts of the small intestines (arrows). Total magnification = 600x.

PANEL C [Figure 4C]. RT-PCR in situ hybridization was performed on serial 10 μm sections of the normal monkey amygdala. Figures 4c1 (300x) and 4c3 (600x) show positive 5-HT3-A mRNA signal (purple in color – dark grey in black-and-white) in seven neurons (arrows) which is also show positive 5HT3-B mRNA signal (4c2 (300x); 4c4 (600x)). Not all cells were labeled by the RT-PCR ISH technique using specific probes for 5-HT3-B or 5-HT3-A.

Figure 5 - PANEL A [Figures 5A and 5B]. Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: 5-HT3-B modifies the kinetics and magnitude of 5-HT3-A currents elicited by a subset of agonists. (a.) 5-HT3-B normalizes agonist-induced 5-HT3-A responses in *Xenopus* oocytes. 5-HT3-A-expressing oocytes responded to 5-HT, 2-methyl-5-HT, mCPBG and 1-PBG with either a slowly desensitizing response (left panel; "slow" responders, 71% of oocyte batches), or a complex current that included a rapidly desensitizing component (right panel; "fast" responders). Oocytes expressing both subunits produced uniform responses (middle panel). The agonists tested included 5-HT (0.3 and 10 μM, responses are superimposed); mCPBG (0.3 and 10 μM); 1-PBG (10 and 100 μM); DA (0.1 and 1 mM); 2-Me-5-HT (10 μM). Agonists are applied during the time indicated by the horizontal bar above the record. The clear bar extending from the solid bar indicates the longer exposure to agonist for one of the superimposed traces. Oocytes were continuously perfused with Ba^{2+} containing saline at a rate of 10 ml/min at room temperature. Time scale bar: 40 sec. (b.) Responses to 1-PBG were blocked by the 5-HT3 antagonist tropisetron. After obtaining a control response to 1-PGB (100 μM; indicated by the solid bar) (left panel), the oocyte was washed 2 min then incubated for 30 sec in tropisetron (1 μM; clear bar) then challenged with both 1-PBG and tropisetron. The response to 1-PBG was completely blocked (middle panel). The response to agonist recovered after a 2 min washout of antagonist (right panel).

PANEL B [Figure 5C]. Peak currents elicited by the indicated agonists from oocytes expressing 5-HT3-A (grey bars) and both 5-HT3-B and 5-HT3-A (solid bars). Data from oocytes recorded in Ba^{2+}- and Ca^{2+}- containing saline were combined since there were no differences in 5-HT (10 μM)-induced maximal current in Ca^{2+}-containing vs. Ba^{2+}-containing saline (-7.7 +/- 1.4 μA (n=16) vs. -7.6 +/- 0.9 μA (n=24)). The number of oocytes tested ranged from 9 to 61; SEM values were 10% for 5-HT (10 μM) and 21% for both mCPBG (10 μM) and 1-PBG (100 μM). p values are indicated: * $p<0.05$, ** $p<0.01$ (Students t-test).

Figure 6 - Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: 5-HT3-B normalized the 5-HT response t80 and time to peak to similar values in "slow" (a,c) and "fast" (b,d) responders. (a.) t80 of responses elicited by 5-HT (concentrations indicated) from experiments in which 5-HT3-A-injected oocytes respond to 5-HT with a slowly decaying response. 5-HT3-A-injected oocytes (grey bars; n were 8, 38 and 13 individual oocytes, respectively); 5-HT3-A receptor and 5-HT3-B -injected oocytes (solid bars; n were 11, 33 and 16 individual oocytes, respectively). The differences were significant at all agonist concentrations ($p< 0.05$, $5e^{-9}$, 0.005 for 1, 10 and 100 μM 5-HT, respectively). (b.) t80 of responses elicited by 5-HT from experiments in which 5-HT3-A-injected oocytes respond to 5-HT with a rapidly decaying response. 5-HT3-A injected oocytes (grey bars; n were 5, 11 and 10 individual oocytes, respectively); 5-HT3-A receptor and 5-HT3-B injected oocytes (solid bars; n were 9, 12 and 3 individual oocytes, respectively). Significant differences were observed at 1 and 10 μM 5-HT ($p < 0.005$ and $p< 5e^{-5}$, respectively). (c.) The time to peak of the response to 10 μM 5-HT in slow responders is significantly faster when 5-HT3-B is co-expressed with 5-HT3-A (solid bars, n= 26) compared to 5-HT3-A alone (grey bars, n=41, $p<0.0005$). (d.) The time to peak of the response to 10 μM 5-HT in "fast" responders was significantly slower

when 5-HT3-B is co-expressed (solid bars, n=12) compared to homomers (grey bars, n=10, $p<0.05$).

Figure 7 - Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: Agonist dose response relationships are altered in the presence of 5-HT3-B and depend on the ratio of 5-HT3-B to 5HT3-A cRNA injected in Xenopus oocytes. (a.) Oocytes were injected with either 5-HT3-A or both 5-HT3-A and 5-HT3-B cRNA and tested for their response to the indicated concentrations of 5-HT. The data are presented relative to the maximum response elicited by 100 μM 5-HT in the same oocytes. 5-HT3-B decreases the apparent affinity of the 5HT3-A receptor for 5-HT. The agonist was applied to the cell at a rate of 10 ml per min in bath perfusate. The data were analyzed using GraphPad Prizm and fit with a Boltzmann exponential. (b.) The percent maximum response for 0.3 μM 5-HT, 0.3 μM mCPBG and 10 μM 1-PBG are plotted for oocytes injected with 5-HT3-A alone (left-most stippled bars), both subunits at a 1:10 ratio (5-HT3-A to 5-HT3-B, solid bars), both subunits at a 1:1 ratio (hatched bars), both subunits at a 1:0.1 ratio (clear bars), and both subunitis at a 1: 0.01 ratio (cross-hatched bars). The percent of the maximal response obtained in individual oocytes was averaged. Significant differences are indicated by asterisks (* $p<0.05$; ** $p< 0.01$).

Figure 8- Specificity of the modulatory effect of human 5-HT3-B in oocytes is shown. In all cases the amount of injected 5-HT3-B cRNA was at least 2-fold the concentration of each nACh receptor. (a.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha3\beta4$ with (solid; n= 5) and without (grey; n=4) 5-HT3-B. (b.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha4\beta2$ with (solid; n=8) and without (grey; n=9) 5-HT3-B. (c.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha2\beta2$ with (solid; n=3) and without (grey; n=3) 5-HT3-B.

(d.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α7 with (solid; n=12) and without (grey; n=15) 5-HT3-B.

Figure 9 - PANEL A [Figures 9A and 9B]. Functional expression of human 5-HT3-B in recombinant host cells is shown: 5-HT3-B and 5-HT3-A heteromers display pharmacological and voltage-dependent properties distinct from 5-HT3-A homomeric receptors. (a.) Ca^{2+} influx induced by agonist challenge was determined using the Ca^{2+} sensitive dye Fluo-4 on the FLIPR system. Responses to 0.17 and 3 μM 5-HT (top), 6 and 30 μM 1-PBG (middle), 0.3 and 3 μM mCPBG are shown. Each record represents 3.33 min. Agonists were added after 20 seconds and were present throughout the recording. Left: 5-HT3-A/HEK cells; Right: 5-HT3-A/5-HT3-B/HEK cells. (b.) Dose response for 5-HT - activated Ca^{2+} influx using the FLIPR system. 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) had significantly higher affinity toward 5-HT and decreased nH compared to 5-HT3-A homomeric receptors (squares). Peak responses were determined and normalized to the maximum observed response. Mean values from 4-10 separate experiments are presented. Data were fit with Boltzmann exponentials.

PANEL B [Figure 9C]. Dose response for mCPBG-activated Ca^{2+} influx using the FLIPR system. Data from 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) and 5-HT3-A homomeric receptors (squares) is shown. Peak responses were determined and normalized to the maximum observed response. Values were averaged from 4 separate experiments. Data were fit with Boltzmann exponentials.

PANEL C [Figures 9D and 9E]. Voltage clamp recordings from 5-HT3-A/HEK cells (left) and 5-HT3-A/5-HT3-B/HEK cells (right). Spikes in the current record are the currents induced by voltage ramp protocols used to determine the change in whole cell membrane conductance. Voltage ramps were

evoked every second. (d.) Top: inward currents elicited by 10 μM 1-PBG (solid bar) and 10 μM 5-HT (clear bar) from a holding potential of -68 mV. Bottom: inward currents elicited by 100 μM 1-PBG (hatched bar). (e.) The voltage relationship for the agonist induced currents obtained using a voltage ramp protocol. 5-HT induced currents from transfected HEK293 cells expressing the homomeric receptor (left) and 5-HT3-A and 5-HT3-B subunits (right). A voltage ramp was applied to the cell from -120 to +80 mV (not corrected for a junction potential of -18 mV) over 200 msec (1 mV/ms) before (solid circle) and during (clear circle) agonist application. The V_{rev} were similar for both responses and were near 0 mV. Asterisks above the recordings in (d) indicate the ramp currents shown on an expanded scale in (e).

DETAILED DESCRIPTION

The present invention relates to DNA encoding human 5-HT3-B, which was isolated from a cDNA library from human small intestine. The human 5-HT3-B, as used herein, refers to protein, which can specifically function as a receptor in a complex with the 5-HT3-A receptor.

The complete amino acid sequence of the human 5-HT3-B was not known, nor was the complete nucleotide sequence encoding human 5-HT3-B known prior to the cloning and functional determination of the present invention. However, the cloning of a full length DNA molecule encoding human 5-HT3-B and some aspects of the function of the protein encoded by this molecule was recently reported (Davies et al., 1999).

The present invention provides a human 5-HT serotonin receptor complex that has distinct pharmacological, kinetic and voltage-dependent properties, which mimic native responses to a greater degree than previously described. Thus the invention described herein shows that 5-HT3-B confers distinct pharmacological, kinetic and voltage-dependent properties upon the 5-HT3-A receptor. The

present invention shows that 5-HT3-B specifically interacts with the 5-HT3-A and not the nicotinic ACh receptors $\alpha 3\beta 4$, $\alpha 2\beta 2$, $\alpha 4\beta 2$ and $\alpha 7$. Furthermore, the mRNA encoding the invention described herein is co-localized within neurons in the monkey amygdala and human cerebral cortex. The present invention further shows that 5-HT3-B and 5-HT3-A mRNA are expressed in lymphocytes and epithelial cells of peripheral organs including the spleen and small intestine. The physiological significance of the novel findings reported herein include the ability of cells co-expressing 5-HT3-B and 5-HT3-A to be more sensitive to 5-HT than cells expressing single receptors, and to have a altered response duration to agonist. These alterations in receptor-mediated current could have profound effects on 5-HT activation of neuronal excitability. It is predicted that a wide variety of cells and cell types will contain the human 5-HT3-B. Vertebrate cells capable of producing human 5-HT3-B include, but are not limited to human 5-HT3-B -expressing cells isolated from cells that show sensitivity to or bind serotonin.

Other cells and cell lines may also be suitable for use to isolate human 5-HT3-B cDNA. Selection of suitable cells may be done by screening for the response to 1-phenylbiguanide (1-PBG) or mCPBG, either the magnitude of the response at low micromolar concentrations of 1-PBG or mCPBG, or the rate of decay of the cellular response elicited by 1-PBG, mCPBG, or serotonin. Human 5-HT3-B activity can be monitored by performing a ^{3}H- [mCPBG] binding assay in the presence of 5-HT3-A receptor (Steward et al., 1993), by direct measurement of a Ca^{+2} influx using the Ca^{+2} sensitive dyes (Kuntzweiler et al., 1998), or by net ion flux using voltage clamp techniques (Hamill et al., 1981). Cells that possess isolate human 5-HT3-B activity in this assay may be suitable for the isolation of isolate human 5-HT3-B DNA or mRNA.

Any of a variety of procedures known in the art may be used to molecularly clone human 5-HT3-B DNA. These methods include, but are not

limited to, direct functional expression of the human 5-HT3-B genes following the construction of a human 5-HT3-B-containing cDNA library in an appropriate expression vector system. Another method is to screen human 5-HT3-B -containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a labelled oligonucleotide probe designed from the amino acid sequence of the human 5-HT3-B subunits. An additional method consists of screening a human 5-HT3-B -containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA encoding the human 5-HT3-B protein. This partial cDNA is obtained by the specific PCR amplification of human 5-HT3-B DNA fragments through the design of degenerate oligonucleotide primers from the amino acid sequence of the purified human 5-HT3-B protein.

Another method is to isolate RNA from human 5-HT3-B -producing cells and translate the RNA into protein via an *in vitro* or an *in vivo* translation system. Translation of the RNA into a peptide a protein will result in the production of at least a portion of the human 5-HT3-B protein which an be identified by, for example, immunological reactivity with an anti- human 5-HT3-B antibody or by biological activity of human 5-HT3-B protein. In this method, pools of RNA isolated from human 5-HT3-B -producing cells can be analyzed for the presence of an RNA that encodes at least a portion of the human 5-HT3-B protein. Further fractionation of the RNA pool can be done to purify the human 5-HT3-B RNA from non- human 5-HT3-B RNA. The peptide or protein produced by this method may be analyzed to provide amino acid sequences which in turn are used to provide primers for production of human 5-HT3-B cDNA, or the RNA used for translation can be analyzed to provide nucleotide sequences encoding human 5-HT3-B and produce probes for this production of human 5-HT3-B cDNA. This method is known in the art and can be found in, for example, Maniatis, T., Fritsch, E.F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989.

It is readily apparent to those skilled in the art that other types of libraries, as well as libraries constructed from other cells or cell types, may be useful for isolating human 5-HT3-B -encoding DNA. Other types of libraries include, but are not limited to, cDNA libraries derived from other cells, from organisms other than human, and genomic DNA libraries that include YAC (yeast artificial chromosome) and cosmid libraries.

It is readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from cells or cell lines which have human 5-HT3-B activity. The selection of cells or cell lines for use in preparing a cDNA library to isolate human 5-HT3-B cDNA may be done by first measuring cell-associated human 5-HT3-B activity using the measurement of human 5-HT3-B -associated biological activity or a human 5-HT3-B - 5-HT3-A receptor ligand binding assay [^{3}H-mCPBG] (Steward et al., 1993).

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Maniatis, T., Fritsch, E.F., Sambrook, J., Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

It is also readily apparent to those skilled in the art that DNA encoding human 5-HT3-B may also be isolated from a suitable genomic DNA library. Construction of genomic DNA libraries can be performed by standard techniques well known in the art. Well known genomic DNA library construction techniques can be found in Maniatis, T., Fritsch, E.F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

In order to clone the human 5-HT3-B gene by the above methods, the amino acid sequence of human 5-HT3-B may be necessary. To accomplish this, human 5-HT3-B protein may be purified and partial amino acid sequence determined by automated sequencers. It is not necessary to determine the entire amino acid sequence, but the linear sequence of two regions of 6 to 8 amino acids from the protein is determined for the production of primers for PCR amplification of a partial human 5-HT3-B DNA fragment.

Once suitable amino acid sequences have been identified, the DNA sequences capable of encoding them are synthesized. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human 5-HT3-B sequence but will be capable of hybridizing to human 5-HT3-B DNA even in the presence of DNA oligonucleotides with mismatches. The mismatched DNA oligonucleotides may still sufficiently hybridize to the human 5-HT3-B DNA to permit identification and isolation of human 5-HT3-B encoding DNA. DNA isolated by these methods can be used to screen DNA libraries from a variety of cell types, from invertebrate and vertebrate sources, and to isolate homologous genes.

Purified biologically active human 5-HT3-B may have several different physical forms. Human 5-HT3-B may exist as a full-length nascent or unprocessed polypeptide, or as partially processed polypeptides or combinations of processed polypeptides. The full-length nascent human 5-HT3-B polypeptide may be posttranslationally modified by specific proteolytic cleavage events that result in the formation of fragments of the full-length nascent polypeptide. A fragment, or physical association of fragments may have the full biological activity associated with human 5-HT3-B however, the degree of human 5-HT3-B

activity may vary between individual human 5-HT3-B fragments and physically associated human 5-HT3-B polypeptide fragments.

The cloned human 5-HT3-B DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant human 5-HT3-B protein. Techniques for such manipulations are fully described in Maniatis, T, et al., supra, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria including E. coli, blue-green algae, plant cells, insect cells, fungal cells including yeast cells, and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungal cells or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one that causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant human 5-HT3-B in mammalian cells. Commercially available

mammalian expression vectors which may be suitable for recombinant human 5-HT3-B expression, include but are not limited to, pMAMneo (Clontech), pcDNA3 (InVitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1 (8-2) (ATCC 37110), pdBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lZD35 (ATCC 37565).

A variety of bacterial expression vectors may be used to express recombinant human 5-HT3-B in bacterial cells. Commercially available bacterial expression vectors that may be suitable for recombinant human 5-HT3-B expression include, but are not limited to pET vectors (Novagen) and pQE vectors (Qiagen).

A variety of fungal cell expression vectors may be used to express recombinant human 5-HT3-B in fungal cells such as yeast. Commercially available fungal cell expression vectors which may be suitable for recombinant human 5-HT3-B expression include but are not limited to pYES2 (InVitrogen) and Pichia expression vector (InVitrogen).

A variety of insect cell expression vectors may be used to express recombinant human 5-HT3-B in insect cells. Commercially available insect cell expression vectors that may be suitable for recombinant expression of human 5-HT3-B include but are not limited to pBlueBacII (InVitrogen).

DNA encoding human 5-HT3-B may be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as E. coli, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to drosophila and silkworm derived cell lines. Cell lines derived

from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), L-cells, and HEK-293 (ATCC CRL1573).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, protoplast fusion, lipofection, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce human 5-HT3-B protein. Identification of human 5-HT3-B expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti- human 5-HT3-B antibodies, and the presence of host cell-associated human 5-HT3-B activity.

Expression of human 5-HT3-B DNA may also be performed using *in vitro* produced synthetic mRNA. Synthetic mRNA or mRNA isolated from human 5-HT3-B producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being generally preferred.

To determine the human 5-HT3-B DNA sequence(s) that yields optimal levels of human 5-HT3-B activity and/or human 5-HT3-B protein, human 5-HT3-B DNA molecules including, but not limited to, the following can be constructed: the full-length open reading frame of the human 5-HT3-B cDNA encoding the 47.9 kDa protein from approximately base 142 to approximately base 1465 (these numbers correspond to first nucleotide of first methionine and last

nucleotide before the first stop codon) and several constructs containing portions of the cDNA encoding human 5-HT3-B protein. All constructs can be designed to contain none, all or portions of the 5' or the 3' untranslated region of human 5-HT3-B cDNA. Human 5-HT3-B activity and levels of protein expression may be determined following the introduction, both in combination with 5-HT3-A or alone, of these constructs into appropriate host cells. Following determination of the human 5-HT3-B DNA cassette yielding optimal expression in transient assays, this human 5-HT3-B DNA construct is transferred to a variety of expression vectors, for expression in host cells including, but not limited to, mammalian cells, baculovirus-infected insect cells, E. coli, and the yeast S. cerevisiae.

Host cell transfectants and microinjected oocytes may be used to assay both the levels of human 5-HT3-B activity and levels of human 5-HT3-B protein by the following methods. In the case of recombinant host cells, this involves the co-transfection of one or possibly two or more plasmids, containing the human 5-HT3-B DNA encoding one or more fragments or subunits and the 5-HT3-A receptor or transfection of the human 5-HT3-B protein into human cell lines expressing the 5-HT3-A receptor. In the case of oocytes, this involves the co-injection of synthetic RNAs for human 5-HT3-B and 5-HT3-A receptor proteins. Following an appropriate period of time to allow for expression, cellular protein is metabolically labelled with, for example ^{35}S-methionine for 24 hours, after which cell lysates and cell culture supernatants are harvested and subjected to immunoprecipitation with polyclonal antibodies directed against the human 5-HT3-B protein.

Other methods for detecting human 5-HT3-B activity involve the direct measurement of human 5-HT3-B activity in whole cells transfected with human 5-HT3-A receptor with or without 5-HT3-B cDNA or oocytes injected with human 5-HT3-A receptor and 5-HT3-B mRNA. Human 5-HT3-B activity is measured by specific ligand binding and biological characteristics of the host cells

expressing human 5-HT3-B DNA. In the case of recombinant host cells expressing human 5-HT3-A receptor and human 5-HT3-B, patch voltage clamp techniques can be used to measure receptor activity and quantitate human 5-HT3-B protein. In the case of oocytes patch clamp as well as two-electrode voltage clamp techniques can be used to measure the decay rate of agonist-induced currents or agonist dose response.

Levels of human 5-HT3-B protein in host cells are quantitated by immunoaffinity. Cells expressing h5-HT5-B can be assayed for the number of cell surface receptor molecules expressed by measuring the amount of radioactive mCPBG binding to cell membranes. Human 5-HT3-B -specific affinity beads or human 5-HT3-B -specific antibodies are used to isolate for example ^{35}S-methionine labelled or unlabelled human 5-HT3-B protein. Labelled human 5-HT3-B protein is analyzed by SDS-PAGE. Unlabelled human 5-HT3-B protein is detected by Western blotting, ELISA or RIA assays employing human 5-HT3-B specific antibodies.

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human 5-HT3-B sequence but will be capable of hybridizing to human 5-HT3-B DNA even in the presence of DNA oligonucleotides with mismatches under appropriate conditions. Under alternate conditions, the mismatched DNA oligonucleotides may still hybridize to the human 5-HT3-B DNA to permit identification and isolation of human 5-HT3-B encoding DNA.

DNA encoding human 5-HT3-B from a particular organism may be used to isolate and purify homologues of human 5-HT3-B from other organisms. To

accomplish this, the first human 5-HT3-B DNA may be mixed with a sample containing DNA encoding homologues of human 5-HT3-B under appropriate hybridization conditions. The hybridized DNA complex may be isolated and the DNA encoding the homologous DNA may be purified therefrom.

It is known that there is a substantial amount of redundancy in the various codons that code for specific amino acids. Therefore, this invention is also directed to those DNA sequences that contain alternative codons that code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein that does not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of human 5-HT3-B is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of human 5-HT3-B. The term "functional derivatives" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" or to "chemical derivatives" of human 5-HT3-B. The term "fragment" is meant to refer to any polypeptide

subset of human 5-HT3-B. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire human 5-HT3-B molecule or to a fragment thereof. A molecule is "substantially similar" to human 5-HT3-B if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical. The term "analog" refers to a molecule substantially similar in function to either the entire human 5-HT3-B molecule or to a fragment thereof.

Following expression of human 5-HT3-B in a recombinant host cell, human 5-HT3-B protein may be recovered to provide human 5-HT3-B in active form. Several serotonin 5-HT3-A receptor purification procedures are available and suitable for use (Fletcher and Barnes, 1997; Fletcher et al., 1998; Lummis and Martin, 1992; Miller et al., 1992). As described above for purification of human 5-HT3-B from natural sources, recombinant human 5-HT3-B may be purified from cell lysates and extracts, or from conditioned culture medium, by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography.

In addition, recombinant human 5-HT3-B can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for full length nascent human 5-HT3-B, polypeptide fragments of human 5-HT3-B or human 5-HT3-B subunits.

Monospecific antibodies to human 5-HT3-B are purified from mammalian antisera containing antibodies reactive against human 5-HT3-B or are prepared as

monoclonal antibodies reactive with human 5-HT3-B using the technique of Kohler and Milstein, *Nature* 256: 495-497 (1975). Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for human 5-HT3-B. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the human 5-HT3-B, as described above. Human 5-HT3-B specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits being preferred, with an appropriate concentration of human 5-HT3-B either with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of peptide encoding a fragment of human 5-HT3-B associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing Corynebacterium parvum and tRNA. The initial immunization consists of human 5-HT3-B peptide in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of the antigen in Freund's incomplete adjuvant by the same route. Booster injections are given at about three-week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about -20°C.

Monoclonal antibodies (mAb) reactive with human 5-HT3-B are prepared by immunizing inbred mice, preferably Balb/c, with human 5-HT3-B peptide.

The mice are immunized by the IP or SC route with about 0.1 mg to about 10 mg, preferably about 1 mg, of human 5-HT3-B peptide in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed above. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 0.1 to about 10 mg of human 5-HT3-B polypeptide in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions that will allow the formation of stable hybridomas. Fusion partners may include, but are not limited to: mouse myelomas P3/NS1/Ag 4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being generally preferred. The antibody producing cells and myeloma cells are fused in polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days 14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using human 5-HT3-B peptide as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a techniques such as the soft agar technique MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

Monoclonal antibodies are produced *in vivo* by injection of pristane primed Balb/c mice, approximately 0.5 ml per mouse, with about 2×10^6 to about 6×10^6 hybridoma cells about 4 days after priming. Ascites fluid is collected at

approximately 8-12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of anti- human 5-HT3-B mAb is carried out by growing the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of human 5-HT3-B in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the above described methods for producing monospecific antibodies may be utilized to produce antibodies specific for human 5-HT3-B polypeptide fragments, or full-length nascent human 5-HT3-B polypeptide, or the individual human 5-HT3-B subunits. Specifically, it is readily apparent to those skilled in the art that monospecific antibodies may be generated which are specific for only the human 5-HT3-B subunit or the fully functional receptor.

Human 5-HT3-B antibody affinity columns can be made by adding the antibodies to Affigel-10 (Bio-Rad), a gel support which is activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activate esters are then quenched with ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous

protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing human 5-HT3-B subunits are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A_{280}) falls to background, then the protein is eluted with 0.23 M glycine-HC1 (pH 2.6). The purified human 5-HT3-B protein is then dialyzed against phosphate buffered saline.

DNA clones, termed p5HT3BR, are identified which encode proteins that, when expressed in any recombinant host, including but not limited to mammalian cells or insect cells or bacteria form a human 5-HT3-B sensitive to serotonin when co-expressed with 5-HT3-A receptor subunits. The expression of human 5-HT3-B DNA results in the reconstitution of the properties observed in oocytes injected with human 5-HT3-B -encoding poly $(A)^+$ RNA together with 5-HT3-A receptor subunits. These include: modification of the 5-HT-, mCPBG and 1-PBG-induced responses compared to those observed for 5-HT3-A homomultimers.

Serotonin is a biogenic amine transmitter that functions in some capacity in many physiological and pathophysiological conditions. Serotonin acts as a neurotransmitter and neuromodulator in the central and peripheral nervous systems, mediates inflammatory and allergic responses, regulates airway function, controls acid secretion in the stomach, regulates cardiovascular function as well as arterial and venous responses and is likely involved in to processes yet to be determined. The serotonin receptors that mediate these include the ligand-gated 5-HT3 receptor. Overlap of 5-HT3-A and 5-HT3-B receptor expression suggests that the putative heteromultimer is involved in central and peripheral nervous system as well as small intestine, thymus, prostate and uterine function. One way to understand which serotonin receptors are involved in these processes is to develop chemical modulators of the receptors as research tools and therapeutic entities. Recombinant host cells expressing the human serotonin 5-HT3-A and human 5-HT3-B receptors can be used to provide materials for a screening

method to identify such agonists and antagonists. As such, this invention of the human serotonin 5-HT3-B subunit directly teaches a way to identify new agonists and antagonists that may prove useful as research tools or may be used as therapeutics to treat disorders directly or indirectly involving serotonin receptors.

The present invention is also directed to methods for screening for compounds that modulate the expression of DNA or RNA encoding human 5-HT3-B as well as the function of human 5-HT3-B protein *in vivo*. Compounds that modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding human 5-HT3-B, or the function of human 5-HT3-B protein. Compounds that modulate the expression of DNA or RNA encoding human 5-HT3-B or the function of human 5-HT3-B protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Modulators identified in this process are useful as therapeutic agents, research tools and diagnostic agents.

Kits containing human 5-HT3-B DNA or RNA, antibodies to human 5-HT3-B, or human 5-HT3-B protein may be prepared. Such kits are used to detect DNA that hybridizes to human 5-HT3-B DNA or to detect the presence of human 5-HT3-B protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic analyses, diagnostic applications, and epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of human 5-HT3-B DNA, human 5-HT3-B RNA or human 5-HT3-B protein. The

recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of human 5-HT3-B. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant human 5-HT3-B protein or anti- human 5-HT3-B antibodies suitable for detecting human 5-HT3-B. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Nucleotide sequences that are complementary to the human 5-HT3-B encoding DNA sequence can be synthesized for antisense therapy. These antisense molecules may be DNA, stable derivatives of DNA such as phosphorothioates or methylphosphonates, RNA, stable derivatives of RNA such as 2'-*O*-alkylRNA, or other human 5-HT3-B antisense oligonucleotide mimetics. Human 5-HT3-B antisense molecules may be introduced into cells by microinjection, liposome encapsulation or by expression from vectors harboring the antisense sequence. Human 5-HT3-B antisense therapy may be particularly useful for the treatment of diseases where it is beneficial to reduce human 5-HT3-B activity.

Human 5-HT3-B gene therapy may be used to introduce human 5-HT3-B into the cells of target organisms. The human 5-HT3-B gene can be ligated into viral vectors that mediate transfer of the human 5-HT3-B DNA by infection of recipient host cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, poliovirus and the like. Alternatively, human 5-HT3-B DNA can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted DNA transfer using ligand-DNA conjugates or adenovirus-ligand-DNA conjugates, lipofection membrane fusion or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* human 5-HT3-B gene therapy.

Human 5-HT3-B gene therapy may be particularly useful for the treatment of diseases where it is beneficial to elevate human 5-HT3-B activity.

Pharmaceutically useful compositions comprising human 5-HT3-B DNA, human 5-HT3-B RNA, or human 5-HT3-B protein, or modulators of human 5-HT3-B receptor activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders in which modulation of human 5-HT3-B -related activity is indicated. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties that are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages defined by routine testing in order to obtain optimal

inhibition of the human 5-HT3-B receptor or its activity while minimizing any potential toxicity. In addition, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds or modulators identified according to this invention as the active ingredient for use in the modulation of human 5-HT3-B receptors can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds or modulators can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. An effective but non-toxic amount of the compound desired can be employed as a serotonin 5-HT3-A/5-HT3-B receptor modulating agent.

The daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per patient, per day. For oral administration, the compositions are preferably provided in the form of scored or unscored tablets containing 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, and 50.0 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.0001 mg/kg to about 100 mg/kg of body weight per day. The range is more particularly from about 0.001 mg/kg to 10 mg/kg of body weight per day. The dosages of the human 5-HT3-B receptor modulators are adjusted when combined to achieve desired effects. On the other hand, dosages of these various

agents may be independently optimized and combined to achieve a synergistic result wherein the pathology is reduced more than it would be if either agent were used alone.

Advantageously, compounds or modulators of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds or modulators for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds or modulators of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

In the methods of the present invention, the compounds or modulators herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include, without limitation, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methylcellulose, agar, bentonite, xanthan gum and the like.

For liquid forms the active drug component can be combined in suitably flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methyl-cellulose and the like. Other dispersing agents that may be employed include glycerin and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations, which generally contain suitable preservatives, are employed when intravenous administration is desired.

Topical preparations containing the active drug component can be admixed with a variety of carrier materials well known in the art, such as, e.g., alcohols, aloe

vera gel, allantoin, glycerine, vitamin A and E oils, mineral oil, PPG2 myristyl propionate, and the like, to form, e.g., alcoholic solutions, topical cleansers, cleansing creams, skin gels, skin lotions, and shampoos in cream or gel formulations.

The compounds or modulators of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

Compounds of the present invention may also be delivered by the use of monoclonal antibodies as individual carriers to which the compound molecules are coupled. The compounds or modulators of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropylmethacryl-amidephenol, polyhydroxy-ethylaspartamidephenol, or polyethyl-eneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds or modulators of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydro-pyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

For oral administration, the compounds or modulators may be administered in capsule, tablet, or bolus form or alternatively they can be mixed in the animals feed. The capsules, tablets, and boluses are comprised of the active ingredient in combination with an appropriate carrier vehicle such as starch, talc, magnesium stearate, or di-calcium phosphate. These unit dosage forms are prepared by intimately mixing the active ingredient with suitable finely-powdered inert ingredients including diluents, fillers,

disintegrating agents, and/or binders such that a uniform mixture is obtained. An inert ingredient is one that will not react with the compounds or modulators and which is non-toxic to the animal being treated. Suitable inert ingredients include starch, lactose, talc, magnesium stearate, vegetable gums and oils, and the like. These formulations may contain a widely variable amount of the active and inactive ingredients depending on numerous factors such as the size and type of the animal species to be treated and the type and severity of the infection. The active ingredient may also be administered as an additive to the feed by simply mixing the compound with the feedstuff or by applying the compound to the surface of the feed. Alternatively the active ingredient may be mixed with an inert carrier and the resulting composition may then either be mixed with the feed or fed directly to the animal. Suitable inert carriers include corn meal, citrus meal, fermentation residues, soya grits, dried grains and the like. The active ingredients are intimately mixed with these inert carriers by grinding, stirring, milling, or tumbling such that the final composition contains from 0.001 to 5% by weight of the active ingredient.

The compounds or modulators may alternatively be administered parenterally via injection of a formulation consisting of the active ingredient dissolved in an inert liquid carrier. Injection may be either intramuscular, intraruminal, intratracheal, or subcutaneous. The injectable formulation consists of the active ingredient mixed with an appropriate inert liquid carrier. Acceptable liquid carriers include the vegetable oils such as peanut oil, cottonseed oil, sesame oil and the like as well as organic solvents such as solketal, glycerol formal and the like. As an alternative, aqueous parenteral formulations may also be used. The vegetable oils are the preferred liquid carriers. The formulations are prepared by dissolving or suspending the active ingredient in the liquid carrier such that the final formulation contains from 0.005 to 10% by weight of the active ingredient.

Topical application of the compounds or modulators is possible through the use of a liquid drench or a shampoo containing the instant compounds or modulators as an aqueous solution or suspension. These formulations generally contain a suspending agent such as bentonite and normally will also contain an antifoaming agent. Formulations containing from 0.005 to 10% by weight of the active ingredient are acceptable. Preferred formulations are those containing from 0.01 to 5% by weight of the instant compounds or modulators.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1:

Cloning of p5HT3BR

cDNA synthesis

First strand synthesis: Approximately 5 μg of human small intestine mRNA (Clontech) was used to synthesize cDNA using the cDNA synthesis kit (Life Technologies). 2 μl of NotI primer adapter was added to 5μl of mRNA and the mixture was heated to 70 ^{0} C for 10 minutes and placed on ice. The following reagents were added on ice: 4μl of 5x first strand buffer (250mM TRIS-HCl (pH8.3), 375mM KCl, 15mMMgCl$_2$), 2μl of 0.1M DTT, 10mM dNTP (nucleotide triphosphates) mix and 1μl of DEPC treated water. The reaction was incubated at 42 ^{0}C for 5minutes. Finally, 5μl of Superscript RT II was added and incubated at 42 °C for 2 more hours. The reaction was terminated on ice.

Second strand synthesis: The first strand product was adjusted to 93μl with water and the following reagents were added on ice: 30 μl of 5x 2nd strand buffer (100 mM TRIS-HCl (pH6.9), 450 mM KCl, 23 mM MgCl$_2$, 0.75 mM β-NAD+, 50mM $(NH_4)_2SO_4$), 3μl of 10 mM dNTP (nucleotide triphosphates), 1μl E. coli DNA

ligase (10units) 1µl RNase H (2units), 4 µl DNA pol I (10 units). The reaction was incubated at 16°C for 2 hours. The DNA from second strand synthesis was treated with T4 DNA polymerase and placed at 16°C to blunt the DNA ends. The double stranded cDNA was extracted with 150 µl of a mixture of phenol and chloroform (1:1, v:v) and precipitated with 0.5 volumes of 7.5 M NH4OAc and 2 volumes of absolute ethanol. The pellet was washed with 70% ethanol and dried down at 37°C to remove the residual ethanol. The double stranded DNA pellet was resuspended in 25 µl of water and the following reagents were added; 10 µl of 5x T4 DNA ligase buffer, 10 µl of Sal1 adapters and 5 µl of T4 DNA ligase. The ingredients were mixed gently and ligated overnight at 16° C. The ligation mix was extracted with phenol:chloroform:isoamyl alcohol, vortexed thoroughly and centrifuged at room temperature for 5 minutes at 14,000 x g to separate the phases. The aqueous phase was transferred to a new tube and the volume adjusted to 100 ml with water. The purified DNA was size selected on a chromaspin 1000 column (Clontech) to eliminate the smaller cDNA molecules. The double stranded DNA was digested with NotI restriction enzyme for 3-4 hours at 37° C. The restriction digest was electrophoresed on a 0.8 % low melt agarose gel. The cDNA in the range of 1-5 KB was cut out and purified using Gelzyme (InVitrogen). The product was extracted with phenol:chloroform and precipitated with NH_4OAc and absolute ethanol. The pellet was washed with 70% ethanol and resuspended in 10 ml of water.

Ligation of cDNA to the Vector: The cDNA was split up into 5 tubes (2µl each) and the ligation reactions were set up by adding 4.5 µl of water, 2 µl of 5x ligation buffer, 1µl of p-Sport vector DNA (cut with Sal-1 / NotI and phosphatase treated) and 0.5 µl of T4 DNA ligase. The ligation was incubated at 40° C overnight.

Introduction of Ligated cDNA into E. coli by Electroporation:

The ligation reaction volume was adjusted to a total volume of 20 µl with water. Five ml of yeast tRNA, 12.5 ml of 7.5M NH_4OAc and 70 ml of absolute ethanol (-20°C) was added. The mixture was vortexed thoroughly, and immediately centrifuged at room temperature for 20 minutes at 14,000 x g. The pellets were washed in 70% ethanol and each pellet was resuspended in 5 ml of water. All 5 ligations (25ml) were pooled and 100µl of DH10B electro-competent cells (Life Technologies) were electroporated with 1 ml of DNA (total of 20 electroporations), then plated out on ampicillin plates to determine the number of recombinants (cfu) per µl. The entire library was seeded into 2 liters of Super-Broth and maxipreps were made using Promega Maxi Prep kit and purified on cesium chloride gradients.

Screening of library:

One-microliter aliquots of the library constructed above were electroporated into Electromax DH10B cells (Life Technologies). The volume was adjusted to 1 ml with SOC media and incubated for 1 hour at 37°C with shaking. The library was then plated out on 50 150cm^2 plates containing LB to a density of 5000 colonies per plate. These were grown overnight at 37°C.

A probe to 5-HT3-B was generated by polymerase chain reaction using the following primer pair:

5' oligo: 5' GAT CTC CCT ACC TCT AAG TG 3' {SEQ.ID. NO.: 1]
3' oligo: 5' AGC ACA CTG GTC TTG AAC AC 3' [SEQ.ID.NO. 2].

Amplification was cycled 35 times using 50-60°C annealing temperature and a human small intestine cDNA as template. The PCR fragment that was generated (400-500 bp) was 32P-labelled using the Klenow fragment of DNA polymerase I and an oligo labeling kit (Pharmacia). The fragment was then cleaned by one passage through a S-200 column (Pharmacia).

The library colonies are lifted on nitrocellulose filters and cross-linked via UV irradiation (Stratagene). Filters were washed three times in buffer (50 mM TRIS, 1 M NaCl, 2mM EDTA, 1% SDS) at 42°C. Filters were then prehybridized in 1:1 Southern Prehyb:Formamide with salmon sperm DNA (50mg, boiled) for 6 hours at 42°C. Filters were then hybridized with the probe ($1x10^6$ counts/ml) overnight. The filters were then washed one time with 2xSSC/0.2%SDS at room temperature for 15 minutes, 2 times with 0.2xSSC/0.1%SDS at 45°C for 30 minutes each. Filters were then wrapped in plastic wrap and exposed to film (Kodak) overnight at -80°C.

Positive clones were identified. Resulting positives were cored from the original plate, incubated in LB for 45 minutes at 37°C and re-plated overnight. The filter lifting/hybridizing/washing/colony picking procedure was replicated until a single clone or clones were isolated, representing an individual cDNA.

From the screen for human novel 5HT3-like receptor, all cDNA clones were isolated and sequenced. One clone, pH3R, contained a 2699 bp insert (Figure 1). This sequence had an apparent open reading frame from nucleotide 299 to 1335 (Figure 2). This open reading frame encoded a protein of 445 amino acids (Figure 3).

EXAMPLE 2

Cloning of 5-HT3-B cDNA into a Mammalian Expression Vector

The 5-HT3-B cDNAs (collectively referred to as p5HT3BR) were cloned into the mammalian expression vector pcDNA3.1zeo(+) (InVitrogen). The 5-HT3-B cDNA clone was isolated from a human small intestine cDNA library. The full-length cDNA was used as the template for PCR using specific primers with BamHI (5'AAC GTT GAA TTC GCC ACC ATG TTG TCA AGT GTA ATG GCT CCC CTG TGG GCC3') [SEQ.ID.NO. 3] and HindIII (5'AAC GTT AAG CTT TCT TAA GTG CCA GCA CAA TTA CTT GAA G 3') [SEQ.ID.NO. 4] sites for

cloning. The PCR product was purified on a column (Wizard PCR DNA purification kit from Promega) and digested with NheI and NotI (NEB) to create cohesive ends. The product was purified by a low melting agarose gel electrophoresis. The pcDNA3.1zeo(+) vector was digested with NheI and NotI enzymes and subsequently purified on a low melt agarose gel. The linear vector was used to ligate to the 5-HT3-B cDNA inserts. Recombinants were isolated, designated 5-HT3-B, and used to transfect mammalian cells stably expressing the human 5-HT3-A receptor transfected in a pCIneo vector (using EcoRI and XbaI cloning sites) (5-HT3-A/HEK293 cells) by electroporation. Stable cell clones were selected by growth in the presence of G418 and zeocin. Single G418/zeocin resistant clones were isolated and shown to contain the intact 5-HT3-B gene. Clones containing the human 5-HT3-B cDNAs were analyzed for p5HT3BR expression by measuring Ca^{2+} influx using Fluo-4 in response to serotonin and 1-PBG (Figure 9 a, b, c). Responses were compared to those obtained from 5-HT3-A-expressing HEK293 cells.

Cells stably expressing human 5-HT3-B together with human 5-HT3-A were used to test for expression human 5-HT3-B and for functional activity. These cells are used to identify and examine other compounds for their ability to modulate, inhibit or activate the human 5-HT3-B. Other cells expressing both 5-HT3-A and 5-HT3-B subunits can be used to identify and examine other compounds for their ability to modulate, inhibit or activate the human 5-HT3-B.

Cassettes containing the human 5-HT3-B cDNA in the positive orientation with respect to the promoter are ligated into appropriate restriction sites 3' of the promoter and identified by restriction site mapping and/or sequencing. These cDNA expression vectors are introduced into fibroblastic host cells for example HEK293 by standard methods including but not limited to electroporation, or chemical procedures (cationic liposomes, DEAE dextran, calcium phosphate).

All of the vectors used for mammalian transient expression can be used to establish stable cell lines expressing the human 5-HT3-B. Unaltered human 5-HT3-B constructs cloned into expression vectors are expected to program host cells to make human 5-HT3-B protein. The transfection host cells include, but are not limited to, HEK293, CV-1-P [Sackevitz et al., Science 238: 1575 (1987)], tk-L [Wigler, et al. Cell 11: 223 (1977)], NS/0, and dHFr- CHO [Kaufman and Sharp, J. Mol. Biol. 159: 601, (1982)].

Co-transfection of any vector containing the human 5-HT3-B cDNA with a drug selection plasmid including, but not limited to G418, zeocin, aminoglycoside phosphotransferase; hygromycin, hygromycin-B phosphotransferase; APRT, xanthine-guanine phosphoribosyl-transferase, will allow for the selection of stably transfected clones. Levels of the human 5-HT3-B are quantitated by the assays described herein.

The human 5-HT3-B cDNA constructs are also ligated into vectors containing amplifiable drug-resistance markers for the production of mammalian cell clones synthesizing the highest possible levels of the human 5-HT3-B. Following introduction of these constructs into cells, clones containing the plasmid are selected with the appropriate agent, and isolation of an over-expressing clone with a high copy number of plasmids is accomplished by selection in increasing doses of the agent.

The expression of recombinant human 5-HT3-B is achieved by transfection of full-length the human 5-HT3-B cDNA into a mammalian host cell.

EXAMPLE 3

Primary Structure of the Human 5-HT3-B Protein

The nucleotide sequences of p5HT3BR revealed single large open reading frame of about 1326 base pairs as shown in Figure 2. The cDNAs have 5' and 3'-

untranslated extensions of about 141 and about 456 nucleotides for p5-HT3-BR. The first in-frame methionine was designated as the initiation codon for an open reading frame that predicts a human 5-HT3-B protein with an estimated molecular mass (M_r) of about 50.3 kDa. The protein contained hydrophobic amino terminal residues with sequences highly predictive of signal cleavage sites that would result in mature proteins initiating at amino acid 22.

The predicted human 5-HT3-B protein was aligned with nucleotide and protein databases and found to be related to the known 5-HT3-A receptors. Approximately 70% of the amino acids in 5-HT3-B were highly conserved, showing at least 44% amino acid identity within the serotonin 5-HT3 family of receptor. The conserved motifs found in this family of receptor, such as the 4 putative transmembrane domains with similar spacing, were also found in the human 5-HT3-B sequence. The identity of the 5-HT3-B receptor with the 5-HT3-A receptor at the nucleotide level was only about 60%. The human 5-HT3-B protein contained the conserved cysteine residues found in the conserved cysteine-cysteine loop that may form the agonist-binding site of ligand-gated ion channels (Lambert et al., 1995). There is strong homology to the proposed ligand recognition site in the first N-terminal loop in the murine 5-HT3-A and the nicotinic AChR α7 [xIWxPDILxxExxD]; the only difference in the shown "consensus" in the 5-HT3-B protein is a conserved change: L119 to I119. The E106 in the 5-HT3-A (murine) is critical for high affinity 5-HT binding (Boess et al., 1997).

Five potential sites of glycosylation (Marshall, 1972) were located at the extracellular amino terminus and 1 potential site for protein kinase C (Woodgett et la., 1986), 3 potential sites for casein kinase II (Pinna, 1990), and 1 site for mammary gland casein kinase were located in the cytoplasmic loop between M3 and M4 as shown in Figure 3.

EXAMPLE 4

Distribution of 5-HT3-B mRNA.

The tissue distribution of 5-HT3-B mRNAs was determined by semi-quantitative PCR. A primer set specific to 5-HT3-B (TGTGTTCAAGACCAGTGTGC [SEQ.ID.NO.8]; TAGCTTTGGAAGAGCAGTCG [SEQ.ID.NO.9]) was used to complete amplification of a portion of the 5-HT3-B mRNA via PCR using cDNAs templates synthesized from poly (A) RNA (Clontech, Palo Alto, CA) which was extracted from various human tissues (tissue types shown in Figure 4a). To gain increased specificity and sensitivity, an oligonucleotide (TGTTGGTCAAATTCCTCCATGATGAGCAGCGTGGTGGACA [SEQ.ID.NO.10]) was phosphorylated using γ-^{32}P-ATP with polynucleotide kinase as described by manufacturer (Amersham Pharmacia Biotech, Piscataway, New Jersey) and annealed to denatured PCR products and resolved by 6% polyacrylamide gel electrophoresis. The subsequent gel was then dried down and imaged (PhosphorImager 445SI, Molecular Dynamics).

As shown in figure 4a, PCR-based tissue distribution analysis reveals that the 5-HT3-B mRNA is expressed in human cerebral cortex including occipital, frontal and temporal regions, amygdala, hippocampus, testis. Very low levels were observed in adrenal gland, bone marrow, lymph node, salivary gland, thyroid. No detectable transcript was observed in heart, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, ovary, small intestine, colon, leukocytes, cerebellum, medulla, spinal cord, putamen, caudate nucleus, corpus callosum, substantia nigra, and thalamus.

In the CNS, the 5-HT3 receptor is found in high density in nuclei of the lower brainstem, area postrema and nucleus of the tractus solitarius. Lower densities of the receptor are found in the cerebral cortex and limbic areas, including the hippocampus. In the periphery, 5-HT3 receptors are located on pre-

and postganglionic neurons of both sensory and enteric nervous systems (Eglen and Bonhaus, 1996). Northern analysis revealed some overlap of 5-HT3-B and 5-HT3-A receptor distributions.

Figure 4b shows RT-PCR in situ hybridization that was performed on normal human tissues of the spleen (b1, b2) and small intestine (b3, b4). Human spleen had intense 5-HT3-B mRNA (b1) and 5HT3-A mRNA (b2) signal (purple in color – dark grey in black-and-white), respectively, in small lymphocytes (arrows). Similarly, intense 5-HT3-B mRNA (b3) and 5HT3-A mRNA (b4) signal (purple) was detected in stromal fibroblasts of the small intestines (arrows). Total magnification = 600x.

Figure 4c shows RT-PCR in situ hybridization that was performed on serial 10 μm sections of the normal monkey amygdala. Figures 4c1 (300x) and 4c3 (600x) show positive 5-HT3-A mRNA signal (purple in color – dark grey in black-and-white) in seven neurons (arrows) which is also show positive 5HT3-B mRNA signal (4c2 (300x); 4c4 (600x)). Not all cells were labeled by the RT-PCR ISH technique using specific probes for 5-HT3-B or 5-HT3-A.

EXAMPLE 5

Characterization of the Function of protein encoded by p5HT3BR in Xenopus oocytes

Xenopus laevis oocytes were prepared and injected using standard methods previously described and known in the art (Fraser et al., 1993). Ovarian lobes from adult female *Xenopus laevis* (Nasco, Fort Atkinson, WI) were teased apart, rinsed several times in nominally Ca-free saline containing (in mM): NaCl 82.5, KCl 2.5, $MgCl_2$ 1, HEPES 5, adjusted to pH 7.0 with NaOH (OR-2), and gently shaken in OR-2 containing 0.2% collagenase Type 1 (ICN Biomedicals, Aurora, Ohio) for 2-5 hours. When approximately 50% of the follicular layers were removed, Stage V and VI oocytes were selected and rinsed in media consisting of 75% OR-2 and 25% ND-

96. The ND-96 contained (in mM): NaCl 100, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, Na pyruvate 2.5, gentamicin (50 ug/ml), adjusted to pH 7.0 with NaOH. The extracellular Ca^{2+} was gradually increased and the cells were maintained in ND-96 for 2-24 hours before injection. For in vitro transcription, pGEM HE (Liman et al., 1992)) containing human 5-HT3-A (Genbank D49394) or 5-HT3-B cDNA was linearized with NheI and transcribed with T7 or SP6 RNA polymerase (Stratagene) in the presence of the cap analog m7G(5')ppp(5')G. The synthesized cRNA was purified with a Sephadex G-50 spin column. Oocytes were injected with 50 nl of the human serotonin 5-HT3-A receptor with or without the 5-HT3-B RNA (0.02 and 0.002-0.2 ng each) or other channel or receptor subunit. Control oocytes were injected with 50 nl of water. Oocytes were incubated for 2-10 days in ND-96 before analysis for expression of the human 5-HT3-B. Incubations and collagenase digestion were carried out at room temperature. Injected oocytes were maintained in 48 well cell culture clusters (Costar, Cambridge, MA) at 18°C. Whole cell agonist-induced currents were measured 1-14 days after injection with a conventional two-electrode voltage clamp (GeneClamp500, Axon Instruments, Foster City, CA) using standard methods previously described and known in the art (Dascal, 1987). The microelectrodes were filled with 3 M KCl, which had resistances of 1 and 2 MΩ. Cells were continuously perfused with ND96 at 10 ml/min at room temperature unless indicated. Membrane voltage was clamped at -88 mV unless indicated.

5-HT (≥100 μM) had no effect in oocytes injected with putative 5-HT3-B subunit alone (n= 4 (3.3 ng cRNA/oocyte), n= 12 (0.33 ng), n= 12 (0.033 ng), and n=3 (0.0033 ng)) indicating there were no endogenous 5-HT-induced currents in the oocytes used in these studies. Oocytes injected with only 5-HT3-B cRNA (3.3 ng) or 5-HT3-B together with nACh β1, β2 and β3 were insensitive to 300 neuroactive compounds at ≥ 100 μM including 5-HT, ACh, histamine, tyramine, tryptamine, tryptophanamide, tryptophan, norepinephrine, octopamine, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid, tryptophol, alpha-methyl serotonin, glutamate, glycine, GABA, β-alanine, taurine, β-phenylethylamine, 5-

hydroxyindolacetic acid, 5-hydroxyindole, 6-hydroxymelatonin, gamma hydroxybutyrate, cis-4 aminocrotonic acid, agmatine, d-cycloserine, N-acetyl-L-cysteine, acetyl-aspartyl-L-glutamic acid, S-α-histamine, N-α-methyl histamine, melatonin, 5-hydroxyindole 2-carboxylic acid, N-acetyl serotonin, and 5-hydroxyindole 3-acetamide. Injection of 5-HT3-B-injected oocytes with bacterial alkaline phosphatase (0.25- 0.3 U) at least 30 min prior to recording was ineffective in conferring sensitivity to these ligands (n=3).

Three characteristics of the 5-HT3-A receptor agonist-induced responses were dramatically altered in 5-HT3-B-injected oocytes. First, the kinetics of the 5-HT-induced response were markedly altered (Figure 5a); second, peak currents were increased (Figure 5c); and third, 5-HT3-B specifically modified the dose-response relationship to 5-HT, the biphenylguanide derivatives mCPBG and 1-PBG, and 2-Me-5-HT, with no detectable effect on the dose-response to dopamine (DA).

Functional expression of human serotonin 5-HT3 receptor modifier subunit 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes is shown in Figure 5. Oocytes were continuously perfused with Ba^{2+} containing ND96 at a rate of 10 ml/min at room temperature. The inward current through 5-HT3-A homomeric receptors declined during the continued presence of 1 to 100 μM 5-HT as shown in Figure 5a, consistent with the desensitization previously described for this receptor (Belelli et al., 1995; Hope et al., 1996; Lankiewicz et al., 1998). 5-HT3-A-expressing oocytes responded to 5-HT, 2-methyl-5-HT, mCPBG and 1-PBG with either a slowly desensitizing response (left panel; "slow" responders, 71% of oocyte batches), or a complex current that included a rapidly desensitizing component (right panel; "fast" responders). Oocytes expressing both subunits produced uniform responses (middle panel). The agonists tested included 5-HT (0.3 and 10 μM, responses are superimposed); mCPBG (0.3 and 10 μM); 1-PBG (10 and 100 μM); DA (0.1 and 1 mM); 2-Me-5-HT (10 μM). Agonists are applied during the time indicated by the horizontal

bar above the record. The clear bar extending from the solid bar indicates the longer exposure to agonist for one of the superimposed traces. Time scale bar: 40 sec.

Oocytes that had been co-injected with equivalent amounts of 5-HT3-A and 5-HT3-B cRNA always responded similarly, despite radical differences in response kinetics of the two populations of control 5-HT3-A-injected oocytes (Table 1). Thus, in "slow" oocytes, near-maximal 5-HT-induced currents decayed 5 times more quickly (Figure 5, left vs. middle panels; Figure 6a; Table 1), while in "fast" oocytes, responses decayed 3.5-fold more slowly (Figure 5, right vs. middle panels; Figure 6b; Table 1). Furthermore, the time to peak for responses to 5-HT (10 μM) was 3.3 +/- 0.3 sec (n= 38) in the presence of 5-HT3-B for all oocytes tested (Figure 6c,d, solid bars, "slow" and "fast" responders). Thus, in the presence of 5-HT3-B, 5-HT3 receptor responses in oocytes were normalized to a moderately transient current. In contrast to the complex response waveforms observed in the absence of 5-HT3-B, inward currents elicited by 10 μM 5-HT in Ba^{2+} ND96 were best fit to a single exponential (τ=4.9 +/- 0.3 sec; n=25) in more than 80% of the oocytes expressing both subunits. This decay constant was significantly slower than the decay of "fast" responses under identical conditions ($p < 5e^{-6}$). In "slow" responders expressing 5-HT3-B, 5-HT, 2-Methyl-5-HT, mCPBG and 1-PBG decreased the t80 values by 5.6-fold, 5-fold, 2.0-fold and 1.7 fold, respectively, suggesting agonist-dependent differences (Table 1). In contrast, the 5-HT3-B modulatory effect on t80 values of "fast" responders to these agonists was about four-fold. Significant differences in t80 values were observed at 1 and 10 μM 5-HT in all oocyte batches. Interestingly, DA responses did not appear to be altered by 5-HT3-B co-expression (Figure 5a).

The response of homomeric and heteromeric receptors to 5-HT was consistent with the activation of non-selective cation channels since the current reversed near 0 mV. The differences in kinetics appeared to be due to alterations in 5-HT3 receptor function and not due to activation of contaminating endogenous Ca^{2+}-activated

chloride channels. While 5-HT3-A receptors are permeable to Ca^{2+} (Davies et al., 1999; Hargreaves et al., 1994; Ronde and Nichols, 1998; Yang, 1990), the influx of Ca^{2+} appears to be insufficient to activate endogenous chloride currents (Gilon and Yakel, 1995; Mair et al., 1998). Furthermore, 5-HT3-B diminishes the Ca^{2+} permeability of the human recombinant receptor (Davies et al., 1999). 5-HT3-B modulation of channel properties were observed in salines deplete of Ca^{2+}, and at membrane potentials near the chloride equilibrium potential.

Responses to 1-PBG were blocked by the 5-HT3 antagonist tropisetron. After obtaining a control response to 1-PGB (100 μM; indicated by the solid bar) (Figure 5b, left panel), the oocyte was washed 2 min then incubated for 30 sec in tropisetron (1 μM; clear bar) then challenged with both 1-PBG and tropisetron. The response to 1-PBG was completely blocked (Figure 5b, middle panel). The response to agonist recovered after a 2 min washout of antagonist (Figure 5b, right panel).

Co-expression of 5-HT3-A together with 5-HT3-B cRNA (solid bars, Figure 5c) significantly increased the maximum response to mCPBG, 1-PBG and 5-HT compared to expression with 5-HT3-A alone (grey bars, Figure 5c). Data from oocytes recorded in Ba^{2+}- and Ca^{2+}- containing saline were combined since there were no differences in 5-HT (10 μM)-induced maximal current in Ca^{2+}-containing vs. Ba^{2+}-containing saline (-7.7 +/- 1.4 μA (n=16) vs. -7.6 +/- 0.9 μA (n=24)). The number of oocytes tested ranged from 9 to 61; SEM values were 10% for 5-HT (10 μM) and 21% for both mCPBG (10 μM) and 1-PBG (100 μM). p values are indicated: * $p<0.05$, ** $p<0.01$ (Students t-test).

The desensitization of the response was quantified by measuring the time between 80% to peak on the rising and falling phases of the response (t80). The rate of decay was slower in salines in which Ca^{2+} was replaced by Ba^{2+}: in Ca^{2+}- and Ba^{2+}-containing saline, t80 was 11.3 +/- 2.6 sec (n= 18) and 30.3 +/- 4.3 sec (n= 31; p<

0.0005), respectively. Under no condition tested was t80 dependent on voltage. The initial decay of the "fast" responders could be fit by a single exponential with τ= 2.0 +/- 0.3 sec (n=8). Our quantification of kinetic parameters determined from oocyte currents provides a means of comparing response durations and onset between populations of oocytes treated under similar experimental conditions. It is clearly not an accurate description of the underlying channel activity because the relatively slow method of application and oocyte geometry precludes rapid exchange of solutions.

The time to peak of the response to 5-HT (10 μM) was 6.2 +/- 0.6 sec (n=41; Figure 6c) and 1.8 +/- 0.2 sec (n=10; Figure 6d) for slow and fast responders, respectively. Similar differences were obtained in response to 100 μM 5-HT. The t80 of homomeric "fast" responses was voltage independent in both in Ca^{2+}- and Ba^{2+}-containing salines similar to the finding with "slow" responses, however, in contrast to the latter, the t80 was similar in the presence and absence of Ca^{2+}.

The pharmacology of 5-HT3 receptors was modified by 5-HT3-B expression similarly in all batches of oocytes tested and the data were combined. The apparent affinity for 5-HT was decreased when 5-HT3-B was co-expressed with 5-HT3-A in oocytes (Figure 7a). On the other hand, oocytes expressing both subunits were more sensitive to application of low concentrations of mCPBG and 1-PBG compared to oocytes expressing 5-HT3-A alone (Figure 5a). Responses to low concentrations of DA were not enhanced by 5-HT3-B. When ratios of peak response to low and high concentrations of agonist were determined, co-injected oocytes had a larger relative response to the biphenylguanide derivatives (1-PBG and mCPBG) but smaller relative response to 5-HT and the magnitude of these differences depended on the relative ratio of cRNAs injected (Figure 7b). Consistent with the t80 value dependence on ratios of injected cRNA, the differences in pharmacology were no longer observed when 5-HT3-B cRNA was 100-fold more dilute than 5-HT3-A cRNA.

The agonists 1-PBG and mCPBG elicited no response in oocytes injected with 0.33 ng 5-HT3-B subunit alone (the concentration injected to give a 1:1 ratio; n=4),

indicating that the increase in 1-PBG and mCPBG responsiveness in co-injected oocytes was not due to a direct activation of 5-HT3-B homomultimers by these agonists. Furthermore, the response to 1-PBG was similarly blocked by tropisetron (1 μM), LY-278,584 maleate (1 μM), d-tubocurarine (30 μM) in a reversible manner. The selective 5-HT_2 receptor antagonist ketanserin (10 μM) had no effect on agonist responses. The 5-HT-induced response in co-injected oocytes was also blocked by these antagonists.

However, 5-HT3-B altered the voltage-dependence of agonist-induced currents such that they were linear rather than inward rectifying. In experiments in which the currents though 5-HT3 receptors were measured at a range of membrane voltages, 5-HT3-A receptors passed more inward current than outward current at voltages negative and positive to the reversal potential, respectively. In the presence of 5-HT3-B, inward and outward currents were similar and the current-voltage relationship was nearly linear. Injection of the 5-HT3-B RNA had no effect on currents through nACh receptors expressed in oocytes (Figure 8) as well as a *Shaker* potassium channel mutant lacking the N-terminal domain responsible for fast inactivation.

The specificity of the modulatory effect of human 5-HT3-B in oocytes is shown in Figure 8. In all cases the amount of injected 5-HT3-B cRNA was at least 2-fold the concentration of each nACh receptor. (a.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α3β4 with (solid; n= 5) and without (grey; n=4) 5-HT3-B. (b.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α4β2 with (solid; n=8) and without (grey; n=9) 5-HT3-B. (c.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α2β2 with (solid; n=3) and without (grey; n=3) 5-HT3-B. (d.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α7 with (solid; n=12) and without (grey; n=15) 5-HT3-B.

Furthermore, the responses to low and high concentrations of epibatidine (0.3 and 10 μM) were similar in α7 nACh-injected oocytes in the presence or absence of 5-HT3-B indicating that the dose response relationship was not appreciably altered.

EXAMPLE 6

Characterization of the Human 5-HT3-B

Human HEK293 cells stably expressing the human 5-HT3-A receptor were transfected with p5HT3BR. One double transfectant was similar to control 5-HT3-A/HEK cells and was usually used as a control. After three days the cells were selected in the presence of neomycin (500 μg/ml) and zeocin (200 μg/ml) and grown through three 1:10 splits for approximately two weeks. Individual colonies were picked and grown in 6-well dishes. Cells were then plated onto 96-well plates (Biocoat, poly-D-lysine coated black/clear plate, Becton Dickinson part # 354640) and grown to confluence for three days. Wells were rinsed with F12/DMEM, then incubated in Fluo-4 (2 μM) with Pluronic acid (20%, 40μl used in 20 ml total volume) for 1 hour at room temperature. Plates were assayed using the FLIPR (Molecular Devices, FL-101). Cells were challenged with agonists (at 3-fold concentration in 40 μl added to 80 μl at a velocity of 50 μl/sec).

The whole cell patch clamp technique (Hamill et al., 1981) was used to record ligand-induced currents from HEK293 stably expressing 5-HT3-A receptor or both the 5-HT3-A receptor and the 5-HT3-B protein maintained for >2 days on 12 mm coverslips. Cells were visualized using a Nikon Diaphot 300 with DIC Nomarski optics. Cells were continuously perfused in a physiological saline (~0.5 ml/min) unless otherwise indicated. The standard physiological saline ("Tyrodes") contained (in mM): 130 NaCl, 4 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, and 10 hemi-Na-HEPES (pH 7.3, 295-300 mOsm as measured using a Wescor 5500 vapor-pressure (Wescor, Inc., Logan, UT). Recording electrodes were fabricated from borosilicate capillary tubing (R6; Garner Glass, Claremont, CA), the tips were coated with dental periphery wax (Miles

Laboratories, South Bend, IN), and had resistances of 1-2 MΩ when containing intracellular saline (in mM: 100 K-gluconate, 25 KCl, 0.483 $CaCl_2$, 3 $MgCl_2$, 10 hemi-Na-HEPES and 1 K_4-BAPTA (100nM free Ca^{2+}); pH 7.4, with dextrose added to achieve 290 mOsm). Liquid junction potentials were -18 mV using standard pipette and bath solutions as determined both empirically and using the computer program JPCalc (Barry, 1994). All voltages shown are corrected for liquid junction potential. Current and voltage signals were detected and filtered at 2 kHz with an Axopatch 1D patch-clamp amplifier (Axon Instruments, Foster City, CA), digitally recorded with a DigiData 1200B laboratory interface (Axon Instruments), and PC compatible computer system and stored on magnetic disk for off-line analysis. Data acquisition and analysis were performed with PClamp software. Slow changes in holding current were detected and filtered at 2 kHz, and recorded with a LPF202A DC amplifier (Warner, Hamden, CT) and VR-10B digital data recorder (Instrutech, Great Neck, NY) onto video tape. The signal was later analyzed at 10 Hz using Axotape software.

The total membrane capacitance (C_m) was determined as the difference between the maximum current after a 30 mV hyperpolarizing voltage ramp from -68 mV generated at a rate of 10 mV/ms and the steady state current at the final potential (-98 mV) (Dubin et al., 1999).

Apparent reversal potentials (V_{rev}) of ligand-induced conductance changes were determined using a voltage-ramp protocol (Dubin et al., 1999). Voltage ramps were applied every 1 second and the resulting whole cell ramp-induced currents were recorded. Usually the voltage was ramped from negative to positive to negative values. The current required to clamp the cells at -68 mV was continuously monitored. Ligand-induced conductances were determined from whole-cell currents elicited by a voltage-ramp protocol in the presence and absence of ligand. Comparison of control ramp currents and those obtained in the presence of ligand reveals the difference between these currents and indicates the effect of the ligand on the channel protein. The voltage at which there was no net ligand-induced current was determined (V_{rev}).

Most values are presented as the arithmetic mean +/- standard error of the mean (S.E.M.).

Agonist-induced Ca^{2+} and ionic current responses had markedly faster decay kinetics in HEK293 cells stably transfected with both 5-HT3-A and 5-HT3-B subunits compared to cells stably expressing 5-HT3-A receptors (Figure 9). Cells expressing both 5-HT3-B and 5-HT3-A (Figure 9a, right) responded to 3 μM 5-HT (top, largest response), 30 μM 1-PBG (middle, largest response) and 3 μM mCPBG (bottom, largest response) with a faster decay compared to 5-HT3-A receptor-expressing cells (Figure 9a, left). These data are similar to those obtained from "slow" oocytes (Figure 5). Superimposed on the Ca^{2+} influx induced by high concentrations of agonist are responses of cells from the same plate to low concentrations of agonist (Figure 9a). As in the oocytes (Figure 7b), responses to low doses of mCPBG and 1-PBG were a larger percentage of the maximum response observed in doubly transfected cells (Figure 9c; $p < 0.005$ at 0.1 and 0.17 μM mCPBG). However, in contrast to the oocyte data (Figure 7a,b), low concentrations of 5-HT also elicited larger responses in double transfectants compared to 5-HT3-A/HEK (Figure 9b; $p < 0.005$ at 60, 100 and 170 nM 5-HT). Complete dose-response relationships for 5-HT and mCPBG (Figure 9b,c) as well as 1-PBG, 2-methyl 5-HT, 5HTQ, quipazine, DA and mCPP indicate a decreased nH and a higher affinity for agonists in 5-HT3-B-expressing cells (5-HT: EC50: 200 +/- 29 nM (n=8) compared to 540 +/- 50 nM (n=7), $p < 0.005$).

Dose response for 5-HT -activated Ca^{2+} influx using the FLIPR system is shown in Figure 9b. 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) had significantly higher affinity toward 5-HT and decreased nH compared to 5-HT3-A homomeric receptors (squares). Peak responses were determined and normalized to the maximum observed response. Mean values from 4-10 separate experiments are presented. Data were fit with Boltzmann exponentials.

Dose response for mCPBG-activated Ca^{2+} influx using the FLIPR system. Data from 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) and 5-HT3-A homomeric receptors (squares) is shown in figure 9c. Peak responses were determined and normalized to the maximum observed response. Values were averaged from 4 separate experiments. Data were fit with Boltzmann exponentials.

Data acquired during fast application of agonist in the FLIPR system measuring receptor-mediated Ca^{2+} influx indicate that all three agonists behaved similarly: cells co-expressing both subunits revealed a shallower dose-response relationship shifted to higher affinity. A likely reason for the difference between the 5-HT dose response in oocytes and in recombinant mammalian cells is that the fast desensitization of the 5-HT response produced an apparent shift in affinity during the relatively slow application of 5-HT in the oocyte studies. This was not observed for 1-PBG and mCPBG, presumably because the responses to these agonists desensitized more slowly (Table 1).

TABLE 1

The t80 of current responses to prolonged exposure to 5-HT3 receptors is altered in *Xenopus* oocytes expressing heteromers of both 5-HT3-A and 5-HT3-B subunits. Mean +/- SEM (n) for peak responses obtained from a holding potential of -70 mV are shown. p values were determined using the Student's *t*-test.

	Oocytes expressing:	5-HT (10 μM)	2-Me-5-HT (10 μM)	mCPBG (10 μM)	1-PBG (100 μM)
Slow control 5-HT response	5-HT3-A	27.1 +/- 3/0 (38)	35 (1)	31.9 +/- 6.3 (9)	31.7 +/- 2.9 (11)
	5-HT3-A + 5-HT3-B	4.6 +/- 0.3 (33) ($p<5e^{-9}$)	7.3 +/- 0.3 (2)	15.8 +/- 1.6 (7) ($p<0.05$)	19.1 +/- 2.1 (10) ($p<0.005$)
Fast control 5-HT response	5-HT3-A	1.6 +/- 0.2 (11)	2.6 +/- 1.4 (2)	3.6 +/- 1.4 (7)	4.3 +/- 1.3 (5)
	5-HT3-A + 5-HT3-B	5.6 +/- 0.6 (12) ($p<5e^{-5}$)	9.7 +.- 1.6 (5) ($p<0.05$)	22.2 +/- 3.2 (11) ($p<1e^{-4}$)	17.5 +/- 1.6 (7) ($p<e^{-4}$)

The differences in kinetics and pharmacology observed in the Ca^{2+} influx experiments were not due to altered intracellular Ca^{2+} buffering in the Ca^{2+}-influx studies. Similar results were observed for 5-HT and 1-PBG-elicited whole-cell currents (Figure 9d). In cells expressing only 5-HT3-A receptors, 10 μM 1-PBG (solid bar) produced a small inward current and increase in conductance (Figure 9d top left). Cells were challenged with a voltage ramp protocol to simultaneously determine whole cell conductance changes (1 Hz); the ramp-induced currents (spikes in Figure 9d) are shown on a faster time scale in Figure 9e. The cell subsequently revealed a large 5-HT response (clear bar indicates when 10 μM 5-HT was applied). In double transfectants, the response to 10 μM 1-PBG was larger in cells that produced comparable 5-HT responses (Figure 9d top right). Top: inward currents elicited by 10 μM 1-PBG (solid bar) and 10 μM 5-HT (clear bar) from a holding potential of -68 mV. Bottom: inward currents elicited by 100 μM 1-PBG (hatched bar). 1-PBG (10 μM) elicited responses that were 3 +/- 1 % (n=7) of the response to 10 μM 5-HT in individual 5-HT3-A/HEK and 10-fold higher (28 and 34%) of the response to 10 μM 5-HT in individual 5-HT3-A/5-HT3-B/HEK cells. The rate of decay of the response to 5-HT (10 μM; Figure 9d top, clear bar) and 1-PBG (30 μM; Figure 9d bottom, hatched bar) was usually accelerated in the presence of 5-HT3-B.

The voltage dependence of the 5-HT induced current was rectifying in 5-HT3-A-expressing cells and more linear in the presence of 5-HT3-B (Figure 9e). 5-HT induced currents from transfected HEK293 cells expressing the homomeric receptor (left) and 5-HT3-A and 5-HT3-B subunits (right). A voltage ramp was applied to the cell from -120 to +80 mV (not corrected for a junction potential of -18 mV) over 200 msec (1 mV/ms) before (solid circle) and during (clear circle) agonist application. The V_{rev} were similar for both responses and were near 0 mV. Asterisks above the recordings in (d) indicate the ramp currents shown on an expanded scale in (e). The ratio of 5-HT induced current measured 50 mV positive and negative from V_{rev} was

calculated as an indication of the degree of rectification. In 5-HT3-A/HEK cells, the inward current was 2-fold larger than the outward current (ratio: 0.53 +/- 0.04; n=10). In cells expressing both subunits, the current-voltage relationship was linear (ratio: 1.07 +/- 0.10; n=5). This rectification difference observed between the two transfectants was statistically different ($p< 0.005$).

In all cell lines tested, the Ca^{+2} influx observed during challenge with agonists at concentrations near their EC50 was completely blocked by the specific 5-HT3 receptor antagonists tropisetron (IC50 ~ 10 nM), LY 278584 (IC50 ~6 nM) and MDL 72222 (IC50 ~ 15 nM). Ketanserin, an antagonist at 5-HT_2 receptors, had no effect on 5-HT or 1-PBG induced Ca^{2+} responses up to 10 μM. Spiperone, an antagonist at 5-HT_{2A} and D_2 DA receptors, appeared to be a partial agonist at the 5-HT3-A receptor at concentrations above 1 μM.

The mechanism underlying the altered kinetics in the presence of 5-HT3-B is not known. In one possibility, 5-HT3-B allosterically modulates the rate of desensitization. If this were the case, then one prediction is a decrease in the peak currents in 5-HT3-B containing receptors compared to homomeric receptors (which was not observed), but this prediction assumes no change in single channel conductance. Heteromeric channels in fact have a significantly larger single channel conductance compared to homomeric channels (Davies et al., 1999). In a second possibility, 5-HT3-B alters the rate for agonist binding such that the first latency is shorter in heteromers compared to homomers and the rate to desensitize is not altered. A similar model was presented for the sodium channel (Aldrich et al., 1983). The time between 80% peak values on the rising and falling phases of the response (t80) appeared to be proportional to the time to peak response, which is consistent with the latter mechanism. In the majority of oocytes, the time to peak was 2-fold faster if 5-HT3-A was co-injected with 5-HT3-B. Furthermore, the altered pharmacology is consistent with a decrease in first latency for channel opening. The effect observed (a decreased nH and a decreased EC_{50} in the presence of 5-HT3-B) may be explained by a loss of negative cooperativity (Liu and Dilger, 1993) when 5-HT3-A subunits

associate with 5-HT3-B subunits, which have no detectable functional binding site for agonists. In this model, binding of one agonist decreases the affinity of other agonist binding sites for agonist in the receptor complex. 5-HT3 receptors reveal a very strong cooperativity—Hill coefficients have been determined from functional studies to be near 2 or 3 (Brown et al., 1998; Sepulveda et al., 1991; Zhong et al., 1999). In a model in which the first latency decreases while the rate of desensitization remains unchanged, there is predicted to be an increased peak current due to channel opening within a shorter time window, which was observed. Single channel analysis may be prohibitive in this system since homomeric receptors have sub-pS single channel conductances (Davies et al., 1999). Only the heteromeric receptors have a large enough single channel conductance to reliably measure single channel openings (Davies et al., 1999).

EXAMPLE 7

Binding assay on human 5-HT3-B and 5-HT3-A co-transfected mammalian cells.

HEK293 cells stably expressing 5-HT3-A receptor with or without human 5-HT3-B can be used in ^{3}H-[mCPBG] binding assays. Equilibrium ligand binding assays can be performed using conventional procedures (Lummis and Baker, 1997; Lummis et al., 1993). Specific ^{3}H-[mCPBG] binding is observed in membrane preparations from 5-HT3 receptor and human 5-HT3-B transfected cells. Oocytes expressing 5-HT3-A and human 5-HT3-B can be used to measure the affinity of binding of other compounds and their ability to displace ^{3}H-[mCPBG] binding.

EXAMPLE 8

Cloning of the human 5-HT3-B cDNA into E. coli Expression Vectors

Recombinant human 5-HT3-B is produced in E. coli following the transfer of the expression cassette into E. coli expression vectors, including but not limited to, the pET series (Novagen). The pET vectors place human 5-HT3-B expression under control of the tightly regulated bacteriophage T7 promoter. Following

transfer of this construct into an E. coli host that contains a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of human 5-HT3-B is induced when an appropriate lac substrate (IPTG) is added to the culture. The levels of expressed human 5-HT3-B are determined by the assays described herein.

The cDNA encoding the entire open reading frame for human 5-HT3-B is inserted into the NdeI site of pET [16]11a. Constructs in the positive orientation are identified by sequence analysis and used to transform the expression host strain BL21. Transformants are then used to inoculate cultures for the production of human 5-HT3-B protein. Cultures may be grown in M9 or ZB media, whose formulation is known to those skilled in the art. After growth to an OD_{600} to approximately 1.5, expression of human 5-HT3-B is induced with 1 mM IPTG for 3 hours at 37°C.

EXAMPLE 9

Cloning of human 5-HT3-B cDNA into a Baculovirus Expression Vector for Expression in Insect Cells

Baculovirus vectors, which are derived from the genome of the AcNPV virus, are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL# 1711). Recombinant baculoviruses expressing human 5-HT3-B cDNA is produced by the following standard methods (InVitrogen Maxbac Manual): the human 5-HT3-B cDNA constructs are ligated into the polyhedrin gene in a variety of baculovirus transfer vectors, including the pAC360 and the BlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA [Kitts, P.A., Nuc. Acid. Res. 18: 5667 (1990)] into Sf9 cells. Recombinant pAC360 viruses are identified by the absence of inclusion bodies in infected cells and recombinant pBlueBac viruses are identified on the basis of ß-galactosidase expression (Summers, M. D.

and Smith, G. E., Texas Agriculture Exp. Station Bulletin No. 1555). Following plaque purification, human 5-HT3-B expression is measured by the assays described herein.

The cDNA encoding the entire open reading frame for human 5-HT3-B is inserted into the BamHI site of pBlueBacII. Constructs in the positive orientation are identified by sequence analysis and used to transfect Sf9 cells in the presence of linear AcNPV mild type DNA.

Authentic, active human 5-HT3-B is found in the cytoplasm of infected cells. Active human 5-HT3-B is extracted from infected cells by hypotonic or detergent lysis.

EXAMPLE 10

Cloning of human 5-HT3-B cDNA into a yeast expression vector

Recombinant human 5-HT3-B is produced in the yeast S. cerevisiae following the insertion of the optimal human 5-HT3-B cDNA cistron into expression vectors designed to direct the intracellular or extracellular expression of heterologous proteins. In the case of intracellular expression, vectors such as EmBLyex4 or the like are ligated to the human 5-HT3-B cistron [Rinas, U. et al., Biotechnology 8: 543-545 (1990); Horowitz B. et al., J. Biol. Chem. 265: 4189-4192 (1989)]. For extracellular expression, the human 5-HT3-B cistron is ligated into yeast expression vectors which fuse a secretion signal (a yeast or mammalian peptide) to the NH_2 terminus of the human 5-HT3-B protein [Jacobson, M. A., Gene 85: 511-516 (1989); Riett L. and Bellon N. Biochem. 28: 2941-2949 (1989)].

These vectors include, but are not limited to pAVE1>6, which fuses the human serum albumin signal to the expressed cDNA [Steep O. Biotechnology 8: 42-46 (1990)], and the vector pL8PL which fuses the human lysozyme signal to the expressed cDNA [Yamamoto, Y., Biochem. 28: 2728-2732)]. In addition,

human 5-HT3-B is expressed in yeast as a fusion protein conjugated to ubiquitin utilizing the vector pVEP [Ecker, D. J., J. Biol. Chem. 264: 7715-7719 (1989), Sabin, E. A., Biotechnology 7: 705-709 (1989), McDonnell D. P., Mol. Cell Biol. 9: 5517-5523 (1989)]. The levels of expressed human 5-HT3-B are determined by the assays described herein.

EXAMPLE 11

Purification of Recombinant human 5-HT3-B

Recombinantly produced human 5-HT3-B may be purified by antibody affinity chromatography.

Human 5-HT3-B antibody affinity columns are made by adding the anti-human 5-HT3-B antibodies to Affigel-10 (Bio-Rad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents and the cell culture supernatants or cell extracts containing solubilized human 5-HT3-B are slowly passed through the column. The column is then washed with phosphate- buffered saline together with detergents until the optical density (A280) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) together with detergents. The purified human 5-HT3-B protein is then dialyzed against phosphate buffered saline.

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WHAT IS CLAIMED IS:

1. An isolated and purified DNA molecule which encodes human 5-HT3-B protein, or a functional derivative thereof, wherein said protein functions as a human serotonin receptor subunit.

2. The isolated and purified DNA molecule of claim 1, having a nucleotide sequence selected from a group consisting of: **(SEQ.ID.NO.:1)**; **(SEQ.ID.NO.:2)**; and functional derivatives thereof.

3. The isolated and purified DNA molecule of claim 1, wherein said DNA molecule is genomic DNA.

4. An expression vector for expression of human 5-HT3-B protein in a recombinant host, wherein said vector contains a recombinant gene encoding human 5-HT3-B protein according to claim 1, or a functional derivative thereof.

5. The expression vector of claim 4, wherein the expression vector contains a cloned gene encoding human 5-HT3-B protein wherein said protein functions as a human serotonin receptor subunit, having a nucleotide sequence selected from a group consisting of: (SEQ.ID.NO.:1); (SEQ.ID.NO.:2); and functional derivatives thereof.

6. The expression vector of claim 4, wherein the expression vector contains genomic DNA encoding human 5-HT3-B protein.

7. A recombinant host cell containing a recombinantly cloned gene encoding human 5-HT3-B protein wherein said protein functions as a human serotonin receptor subunit, or functional derivative thereof.

8. The recombinant host cell of claim 7, wherein said gene has a nucleotide sequence selected from a group consisting of: (SEQ.ID.NO.:1); (SEQ.ID.NO.:2); and functional derivatives thereof.

9. The recombinant host cell of claim 7, wherein said cloned gene encoding human 5-HT3-B protein is genomic DNA.

10. A protein, in substantially pure form which functions as human 5-HT3-B protein and wherein said protein functions as a modifier of the human 5-HT3-A receptor.

11. The protein according to claim 10, having an amino acid sequence selected from a group consisting of: (SEQ.ID.NO.:3); (SEQ.ID.NO.:4); and functional derivatives thereof.

12. A monospecific antibody immunologically reactive with human 5-HT3-B protein wherein said protein functions as a modifier of the human 5-HT3-A receptor.

13. The antibody of Claim 12, wherein the antibody blocks activity of the 5-HT3-B subunit of the human serotonin receptor.

14. A process for expression of human 5-HT3-B protein in a recombinant host cell, comprising:

(a) transferring the expression vector of Claim 4 into suitable host cells; and

(b) culturing the host cells of step (a) under conditions which allow expression of the human 5-HT3-B protein from the expression vector.

15. A method of identifying compounds that modulate human 5-HT3-B protein activity, comprising:

(a) combining a modulator of human 5-HT3-B protein activity with human 5-HT3-B protein wherein said protein optionally functions as a modifier of the human 5-HT3-A receptor; and

(b) measuring an effect of the modulator on the protein.

16. The method of claim 15, wherein the effect of the modulator on the protein is inhibiting or enhancing binding of human 5-HT3 receptor ligands.

17. The method of claim 15, wherein the effect of the modulator on the protein is stimulation or inhibition of human 5-HT3-B-containing serotonin receptor.

18. The method of claim 17, wherein the human 5-HT3-B is altering the kinetics and the pharmacology of the human 5-HT3-B-containing serotonin receptor.

19. A compound active in the method of Claim 15, wherein said compound is a modulator of a human 5-HT3-B containing serotonin receptor.

20. A compound active in the method of Claim 15, wherein said compound is an agonist or antagonist of a subclass of 5-HT3 receptor consisting of both 5-HT3-A and 5HT3B proteins.

21. A compound active in the method of Claim 15, wherein said compound is a modulator of expression of a 5-HT3-B subunit.

22. A pharmaceutical composition comprising a compound active in the method of Claim 15, wherein said compound is a modulator of human 5-HT3-B subunit activity.

23. A method of treating a patient in need of such treatment for a condition which is mediated by a human 5-HT3-B-containing serotonin receptor , comprising administration of a human 5-HT3-B modulating compound active in the method of Claim 15.

5

FIG. 1

[SEQ.ID.NO.:5]
Nucleic Acid sequence of the human 5-HT3-B
(full sequence including untranslated regions;
1923 bases. (141 bases of 5' UTR; 456 bases of 3' UTR)

CCACGCGTCCGTAAGGATAGCATCAACTGGCAAACGGAGAAGGAGGAGAA
CAGAGTGGAGAGGAACCCTGTTAGGAGAAATTGAGCGGCATTCCATCTGG
TAGGCAAGTTTGCATTTCTCCTTTTTGGGATCTGCCCAGGA**ATG**TTGTCA
AGTGTAATGGCTCCCCTGTGGGCCTGCATCCTGGTGGCTGCAGGAATTCT
AGCCACAGATACACATCATCCCCAGGATTCTGCTCTGTATCATCTCAGCA
AGCAGCTATTACAGAAATATCATAAAGAAGTGAGACCTGTTTACAACTGG
ACCAAGGCCACCACAGTCTACCTGGACCTGTTCGTCCATGCTATATTGGA
TGTGGATGCAGAGAATCAAATATTAAAGACAAGTGTATGGTACCAAGAGG
TCTGGAATGATGAATTTTTATCCTGGAACTCCAGCATGTTTGATGAGATT
AGAGAGATCTCCCTACCTCTAAGTGCCATCTGGGCCCCCGATATCATCAT
CAATGAGTTTGTGGACATTGAAAGATACCCTGACCTTCCCTATGTTTATG
TGAACTCATCTGGGACCATTGAGAACTATAAGCCCATCCAGGTGGTCTCT
GCGTGCAGTTTAGAGACATATGCTTTTCCATTTGATGTCCAGAATTGCAG
CCTGACCTTCAAGAGCATTCTGCATACAGTGGAAGACGTAGACCTGGCCT
TTCTGAGGAGCCCAGAAGACATTCAGCATGACAAAAAGGCGTTTTTGAAT
GACAGTGAGTGGGAACTTCTATCTGTGTCCTCCACATACAGCATCCTGCA
GAGCAGCGCTGGAGGATTTGCACAGATTCAGTTTAATGTGGTGATGCGCA
GGCACCCCCTGGTCTATGTCGTGAGTCTGCTGATTCCTAGCATCTTTCTC
ATGCTGGTGGACCTGGGGAGCTTCTACCTGCCACCCAACTGCCGAGCCAG
GATTGTGTTCAAGACCAGTGTGCTGGTGGGCTACACCGTCTTCAGGGTCA
ACATGTCCAACCAGGTGCCACGGAGTGTAGGGAGCACCCCTCTGATTGGG
CACTTCTTCACCATCTGCATGGCCTTCTTGGTTCTCAGCTTAGCTAAGTC
CATCGTGTTGGTCAAATTCCTCCATGATGAGCAGCGTGGTGGACAGGAGC
AGCCCTTCTTGTGCCTTCGAGGGGACACCGATGCTGACAGGCCTAGAGTG
GAACCCAGGGCCCAACGTGCTGTGGTAACAGAGTCCTCGCTGTATGGAGA
GCACCTGGCCCAGCCAGGAACCCTGAAGGAAGTCTGGTCGCAGCTTCAAT
CTATCAGCAACTACCTCCAAACTCAGGACCAGACAGACCAACAGGAGGCA
GAGTGGCTGGTCCTCCTGTCCCGCTTTGACCGACTGCTCTTCCAAAGCTA
CCTTTTCATGCTGGGGATCTACACCATCACTCTGTGCTCCCTCTGGGCAC
TGTGGGGCGGCGTG**TGA**AGACTGAAGTGTTCTTCAGTAATTGTGCTGGCA
CTTAGGAGAGAGAGAGGGGGAATAATAGTGGGTTAAAAAGCTTTCTGGGT
CGGGTGTGGTGGTTCTTGCCTATAGTCCCAGTGCTTTGGGAGGCCATAGC
AGGAGGATTGCTTGAGCCCAGGAGTTCGAGACCAGCCAGAGCAACATAGT
GAGACCACATCTCTACCAGTAAATAAATAAATAAATAAATAAATAAATAA
ATAAATAAATAGCTGGGCATAGTGGCTCATGCCTGTACTCTCAGCTACTT
GGGAGGTTGAGGTGGGAGGATTGCTTGAGCCCAGGATTTCAAGGCTGCAG
TGAGCCATGATTGCACCACTGCACCCCAGCCTGGGTGACAGAACAAGACC
CTGTCTCAAAAAAAAAATAAAATAAAAGGCTTTCTGCCTTCAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAG

FIG. 2

[SEQ.ID.NO.:6]
Nucleotide sequence for the coding region of 5-HT3-B is shown; 1326 bases.

ATGTTGTCAAGTGTAATGGCTCCCCTGTGGGCCTGCATCCTGGTGGCTGC
AGGAATTCTAGCCACAGATACACATCATCCCCAGGATTCTGCTCTGTATC
ATCTCAGCAAGCAGCTATTACAGAAATATCATAAAGAAGTGAGACCTGTT
TACAACTGGACCAAGGCCACCACAGTCTACCTGGACCTGTTCGTCCATGC
TATATTGGATGTGGATGCAGAGAATCAAATATTAAAGACAAGTGTATGGT
ACCAAGAGGTCTGGAATGATGAATTTTTATCCTGGAACTCCAGCATGTTT
GATGAGATTAGAGAGATCTCCCTACCTCTAAGTGCCATCTGGGCCCCCGA
TATCATCATCAATGAGTTTGTGGACATTGAAAGATACCCTGACCTTCCCT
ATGTTTATGTGAACTCATCTGGGACCATTGAGAACTATAAGCCCATCCAG
GTGGTCTCTGCGTGCAGTTTAGAGACATATGCTTTTCCATTTGATGTCCA
GAATTGCAGCCTGACCTTCAAGAGCATTCTGCATACAGTGGAAGACGTAG
ACCTGGCCTTTCTGAGGAGCCCAGAAGACATTCAGCATGACAAAAAGGCG
TTTTTGAATGACAGTGAGTGGGAACTTCTATCTGTGTCCTCCACATACAG
CATCCTGCAGAGCAGCGCTGGAGGATTTGCACAGATTCAGTTTAATGTGG
TGATGCGCAGGCACCCCCTGGTCTATGTCGTGAGTCTGCTGATTCCTAGC
ATCTTTCTCATGCTGGTGGACCTGGGGAGCTTCTACCTGCCACCCAACTG
CCGAGCCAGGATTGTGTTCAAGACCAGTGTGCTGGTGGGCTACACCGTCT
TCAGGGTCAACATGTCCAACCAGGTGCCACGGAGTGTAGGGAGCACCCCT
CTGATTGGGCACTTCTTCACCATCTGCATGGCCTTCTTGGTTCTCAGCTT
AGCTAAGTCCATCGTGTTGGTCAAATTCCTCCATGATGAGCAGCGTGGTG
GACAGGAGCAGCCCTTCTTGTGCCTTCGAGGGGACACCGATGCTGACAGG
CCTAGAGTGGAACCCAGGGCCCAACGTGCTGTGGTAACAGAGTCCTCGCT
GTATGGAGAGCACCTGGCCCAGCCAGGAACCCTGAAGGAAGTCTGGTCGC
AGCTTCAATCTATCAGCAACTACCTCCAAACTCAGGACCAGACAGACCAA
CAGGAGGCAGAGTGGCTGGTCCTCCTGTCCCGCTTTGACCGACTGCTCTT
CCAAAGCTACCTTTTCATGCTGGGGATCTACACCATCACTCTGTGCTCCC
TCTGGGCACTGTGGGGCGGCGTGTGA

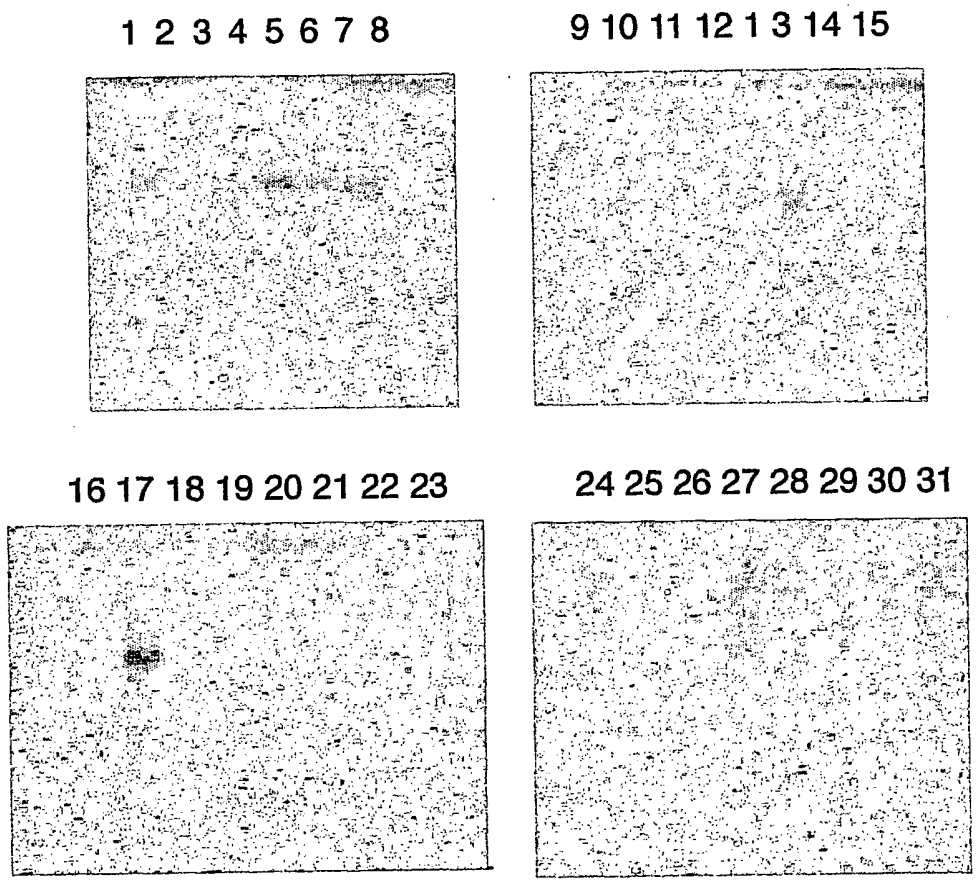
FIG. 3

[SEQ.ID.NO.:7]
The amino acid sequence of 5-HT3-B is shown (441 amino acids).

Met Leu Ser Ser Val Met Ala Pro Leu Trp Ala Cys Ile Leu Val Ala Ala Gly Ile
Leu Ala Thr Asp Thr His His Pro Gln Asp Ser Ala Leu Tyr His Leu Ser
Lys Gln Leu Leu Gln Lys Tyr His Lys Glu Val Arg Pro Val Tyr Asn Trp Thr
Lys Ala Thr Thr Val Tyr Leu Asp Leu Phe Val His Ala Ile Leu Asp Val Asp
Ala Glu Asn Gln Ile Leu Lys Thr Ser Val Trp Tyr Gln Glu Val Trp Asn Asp
Glu Phe Leu Ser Trp Asn Ser Ser Met Phe Asp Glu Ile Arg Glu Ile Ser Leu
Pro Leu Ser Ala Ile Trp Ala Pro Asp Ile Ile Ile Asn Glu Phe Val Asp Ile
Glu Arg Tyr Pro Asp Leu Pro Tyr Val Tyr Val Asn Ser Ser Gly Thr Ile Glu
Asn Tyr Lys Pro Ile Gln Val Val Ser Ala Cys Ser Leu Glu Thr Tyr Ala Phe
Pro Phe Asp Val Gln Asn Cys Ser Leu Thr Phe Lys Ser Ile Leu His Thr Val
Glu Asp Val Asp Leu Ala Phe Leu Arg Ser Pro Glu Asp Ile Gln His Asp Lys
Lys Ala Phe Leu Asn Asp Ser Glu Trp Glu Leu Leu Ser Val Ser Ser Thr Tyr
Ser Ile Leu Gln Ser Ser Ala Gly Gly Phe Ala Gln Ile Gln Phe Asn Val Val
Met Arg Arg His Pro Leu Val Tyr Val Val Ser Leu Leu Ile Pro Ser Ile Phe
Leu Met Leu Val Asp Leu Gly Ser Phe Tyr Leu Pro Pro Asn Cys Arg Ala Arg
Ile Val Phe Lys Thr Ser Val Leu Val Gly Tyr Thr Val Phe Arg Val Asn Met
Ser Asn Gln Val Pro Arg Ser Val Gly Ser Thr Pro Leu Ile Gly His Phe Phe
Thr Ile Cys Met Ala Phe Leu Val Leu Ser Leu Ala Lys Ser Ile Val Leu Val
Lys Phe Leu His Asp Glu Gln Arg Gly Gly Gln Glu Gln Pro Phe Leu Cys Leu
Arg Gly Asp Thr Asp Ala Asp Arg Pro Arg Val Glu Pro Arg Ala Gln Arg Ala
Val Val Thr Glu Ser Ser Leu Tyr Gly Glu His Leu Ala Gln Pro Gly Thr Leu
Lys Glu Val Trp Ser Gln Leu Gln Ser Ile Ser Asn Tyr Leu Gln Thr Gln Asp
Gln Thr Asp Gln Gln Glu Ala Glu Trp Leu Val Leu Leu Ser Arg Phe Asp Arg
Leu Leu Phe Gln Ser Tyr Leu Phe Met Leu Gly Ile Tyr Thr Ile Thr Leu Cys
Ser Leu Trp Ala Leu Trp Gly Gly Val

FIG. 4A

The tissue distribution of 5-HT3-B is shown.



1, Cerebellum; 2, Cerebral cortex; 3, Medulla;
4, Spinal Cord; 5, Occipital pole; 6, frontal lobe;
7, Temporal lobe; 8, Putamen; 9, Amygdala;
10, Caudate nucleus; 11, Corpus callosum; 12, Hippocampus;
13, Whole brain; 14, Substantia nigra; 15, Thalamus;
16, Heart; 17, Brain; 18, Placenta; 19, Lung; 20, Liver;
21, Skeletal muscle; 22, Kidney; 23, Pancreas;
24, Spleen; 25, Thymus; 26, Prostate; 27, Testis;
28, Ovary; 29, Small Intestine; 30, Colon (mucosal lining);
31, Peripheral blood leukocyte.

FIG. 4B

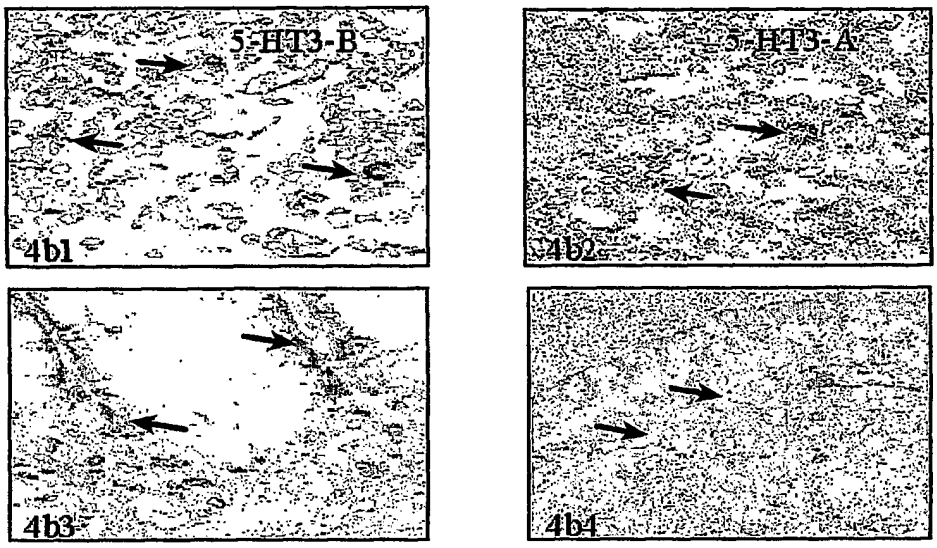


FIG. 4C

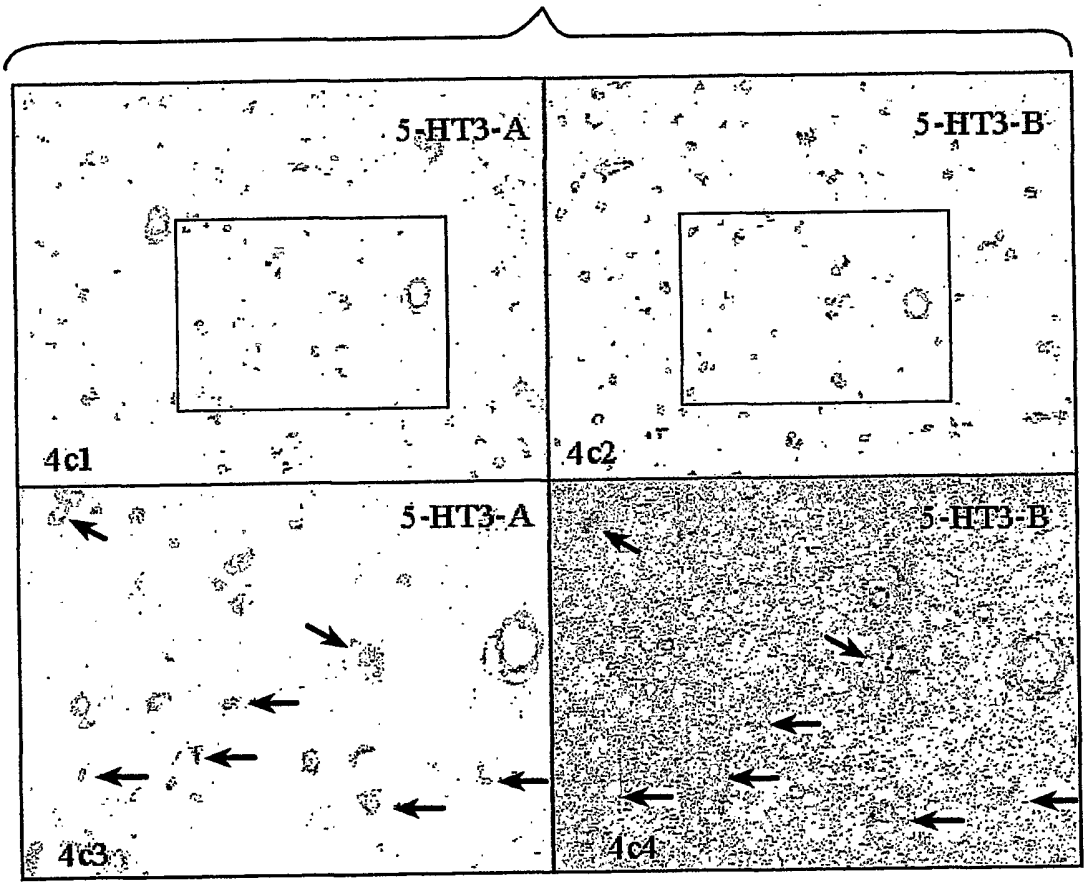


FIG. 5A

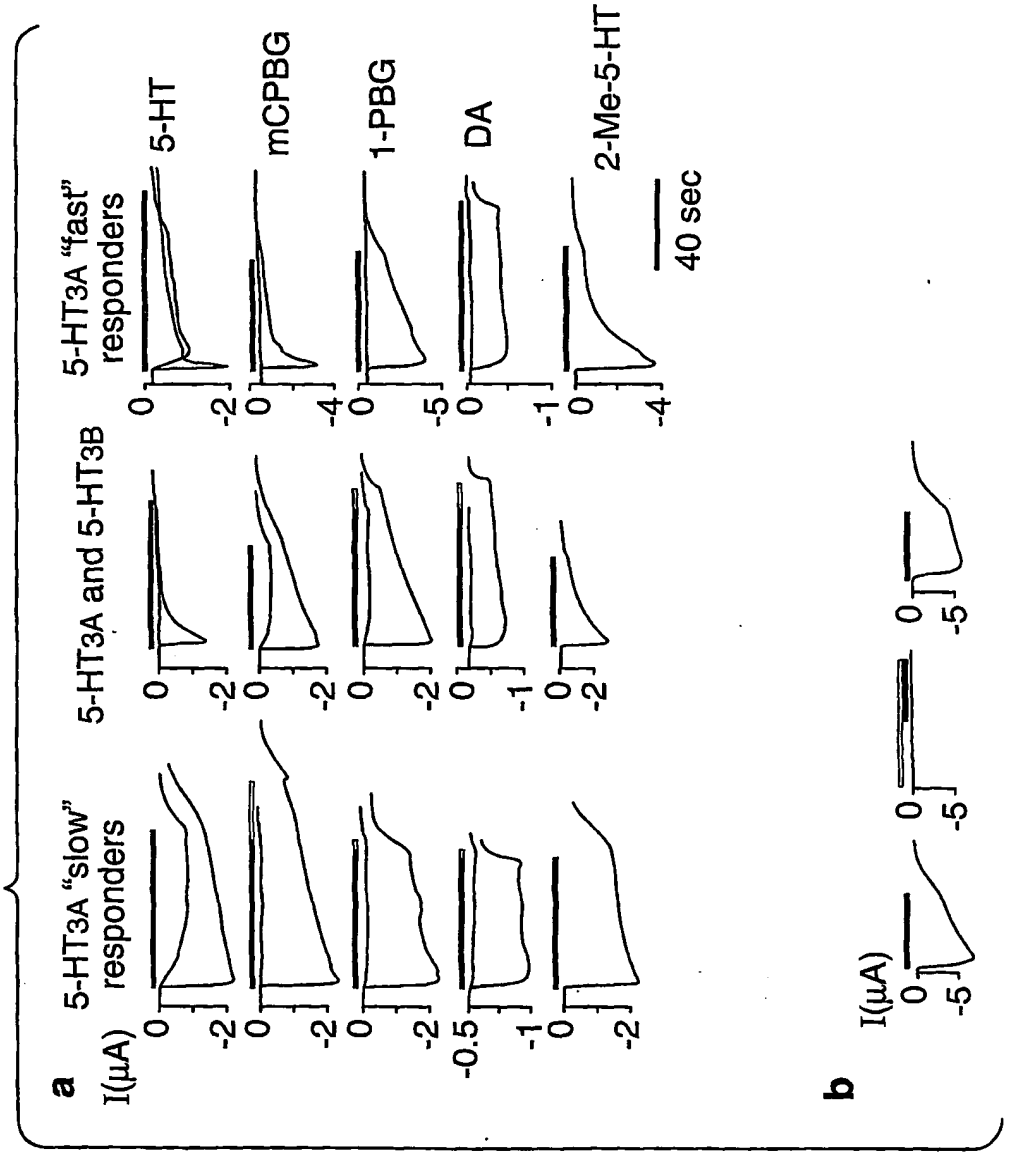

a
I(μA)
5-HT3A "slow" responders
5-HT3A and 5-HT3B
5-HT3A "fast" responders
5-HT
mCPBG
1-PBG
DA
2-Me-5-HT
40 sec
b
I(μA)

FIG. 5B

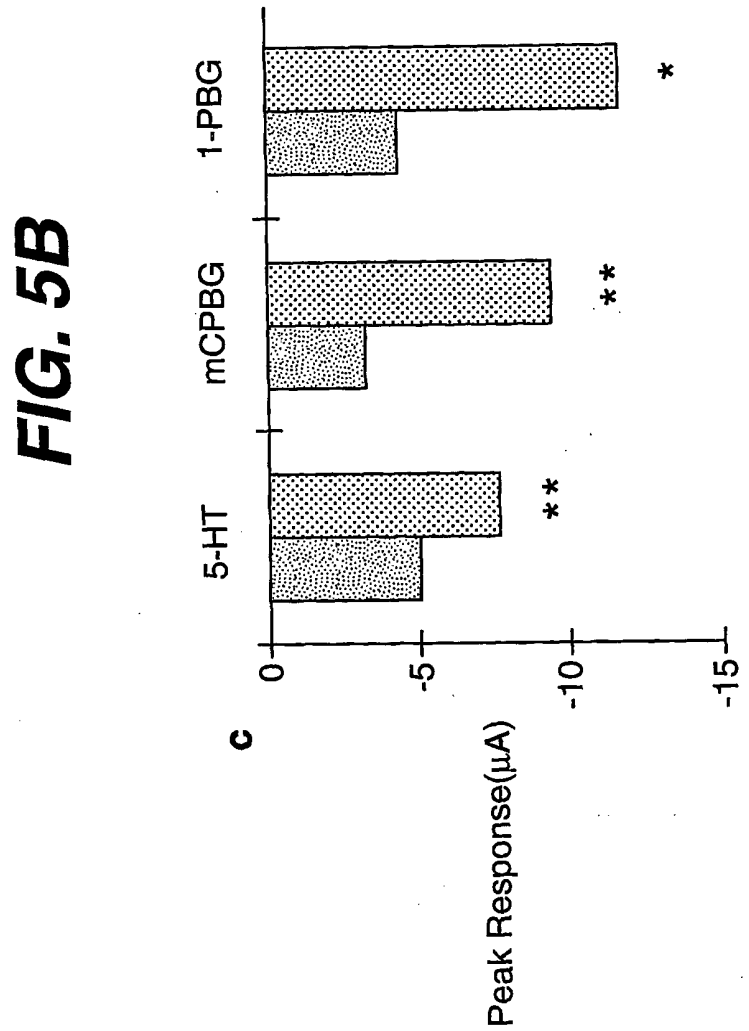


FIG. 6

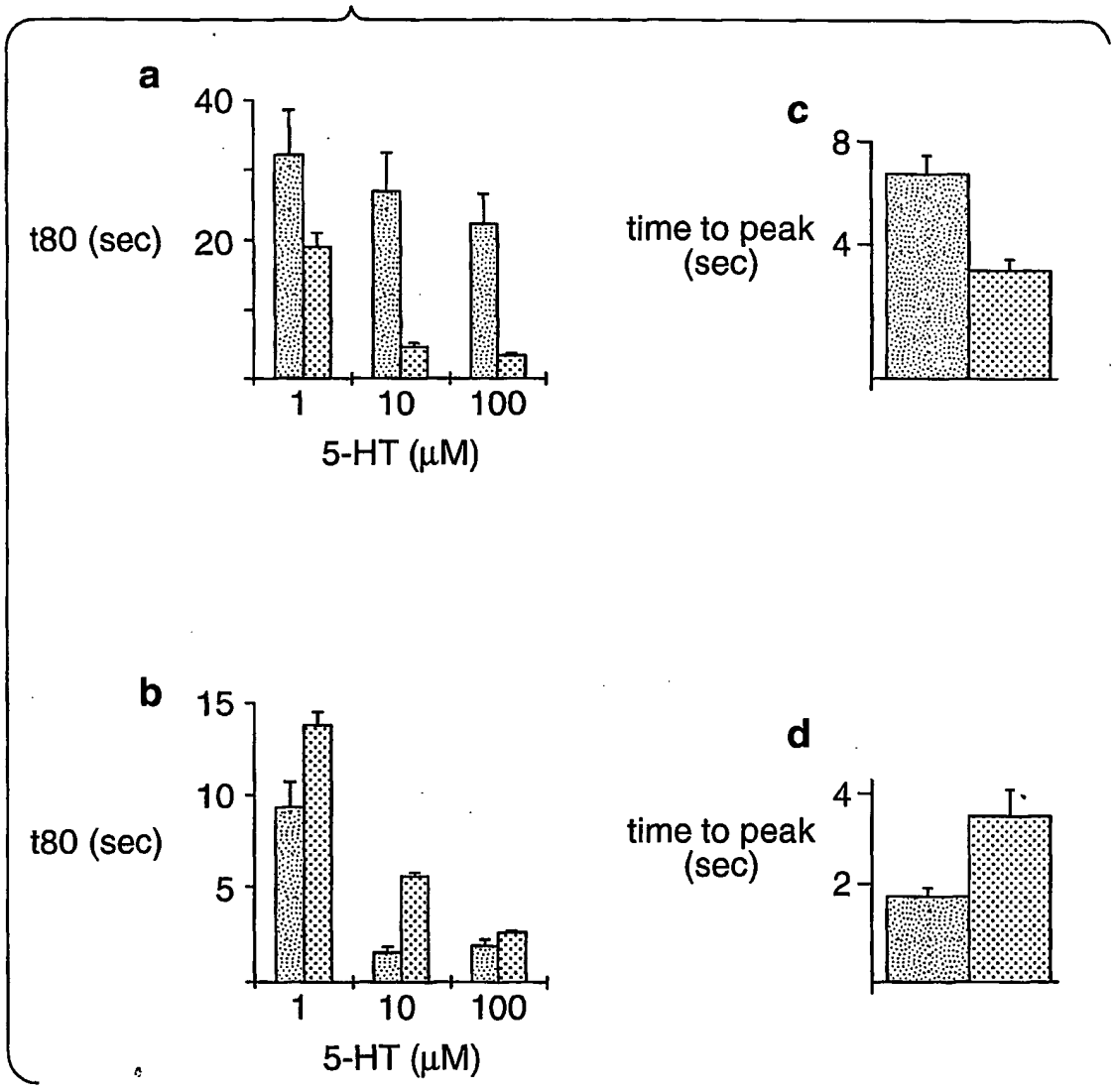

a
40
20
t80 (sec)
1
10
100
5-HT (μM)
c
8
4
time to peak
(sec)
b
15
10
5
t80 (sec)
1
10
100
5-HT (μM)
d
4
2
time to peak
(sec)

FIG. 7

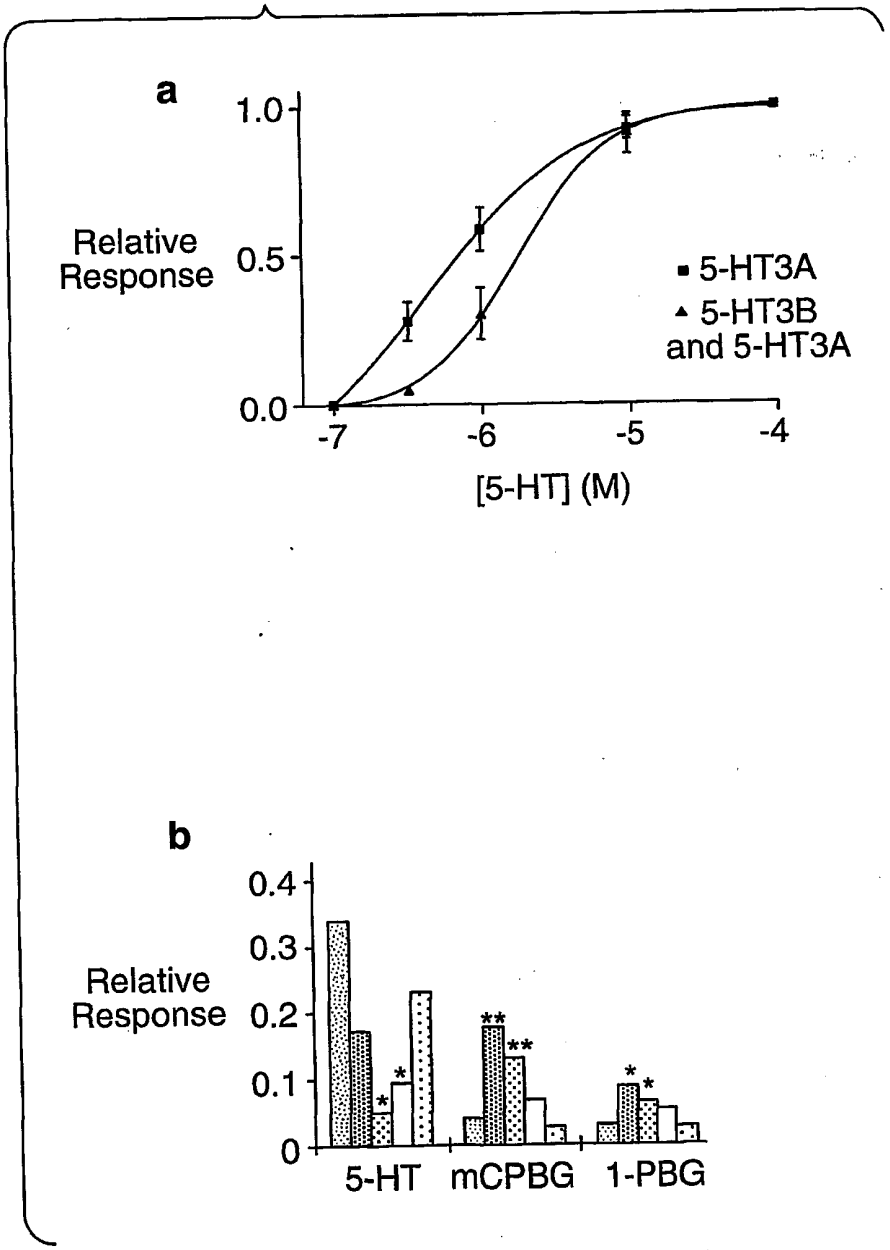

a
1.0
0.5
0.0
Relative Response
-7
-6
-5
-4
[5-HT] (M)
5-HT3A
5-HT3B and 5-HT3A
b
0.4
0.3
0.2
0.1
0
Relative Response
5-HT
mCPBG
1-PBG

FIG. 8

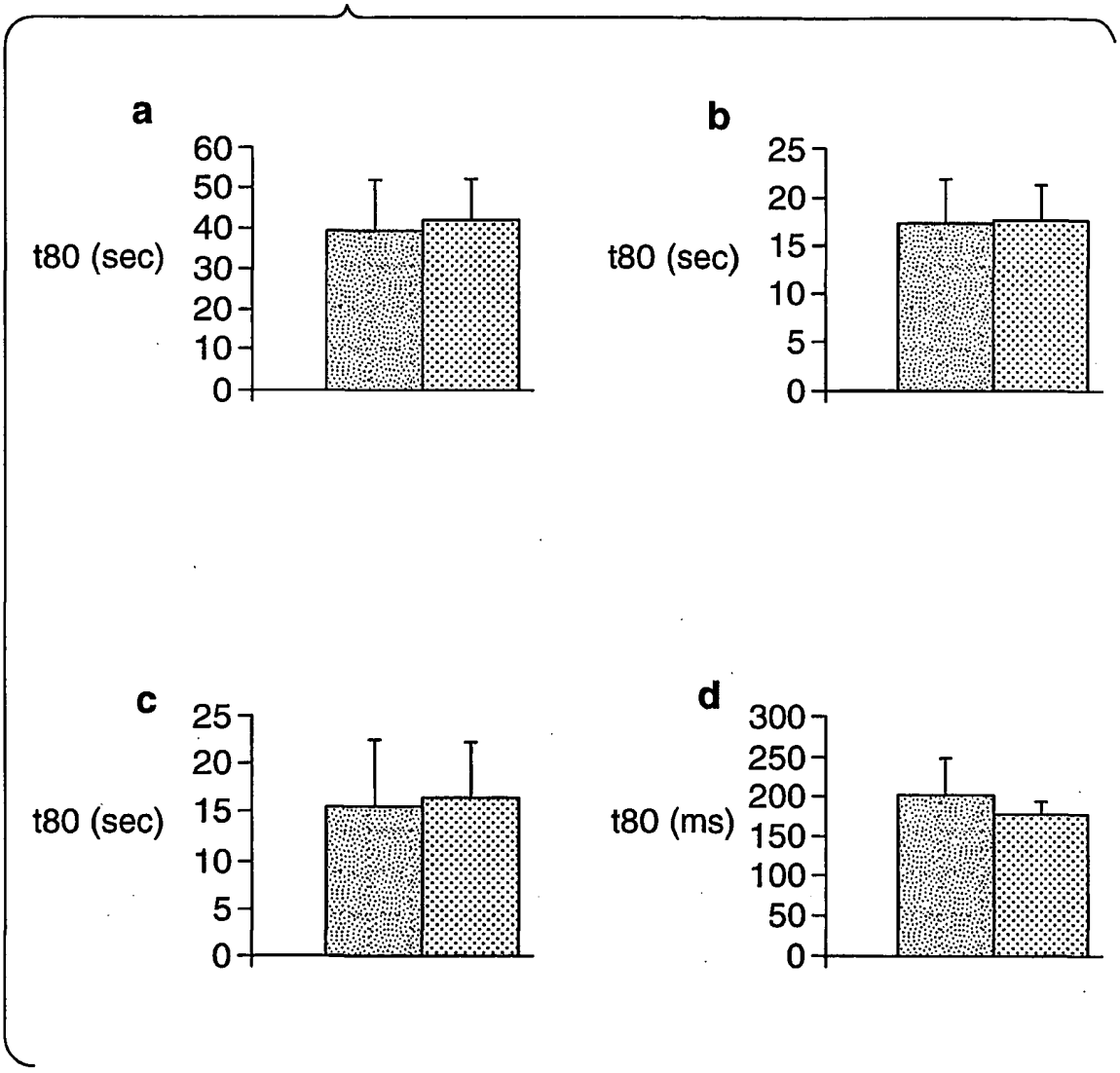

a
t80 (sec)
60
50
40
30
20
10
0
b
t80 (sec)
25
20
15
10
5
0
c
t80 (sec)
25
20
15
10
5
0
d
t80 (ms)
300
250
200
150
100
50
0

FIG. 9A

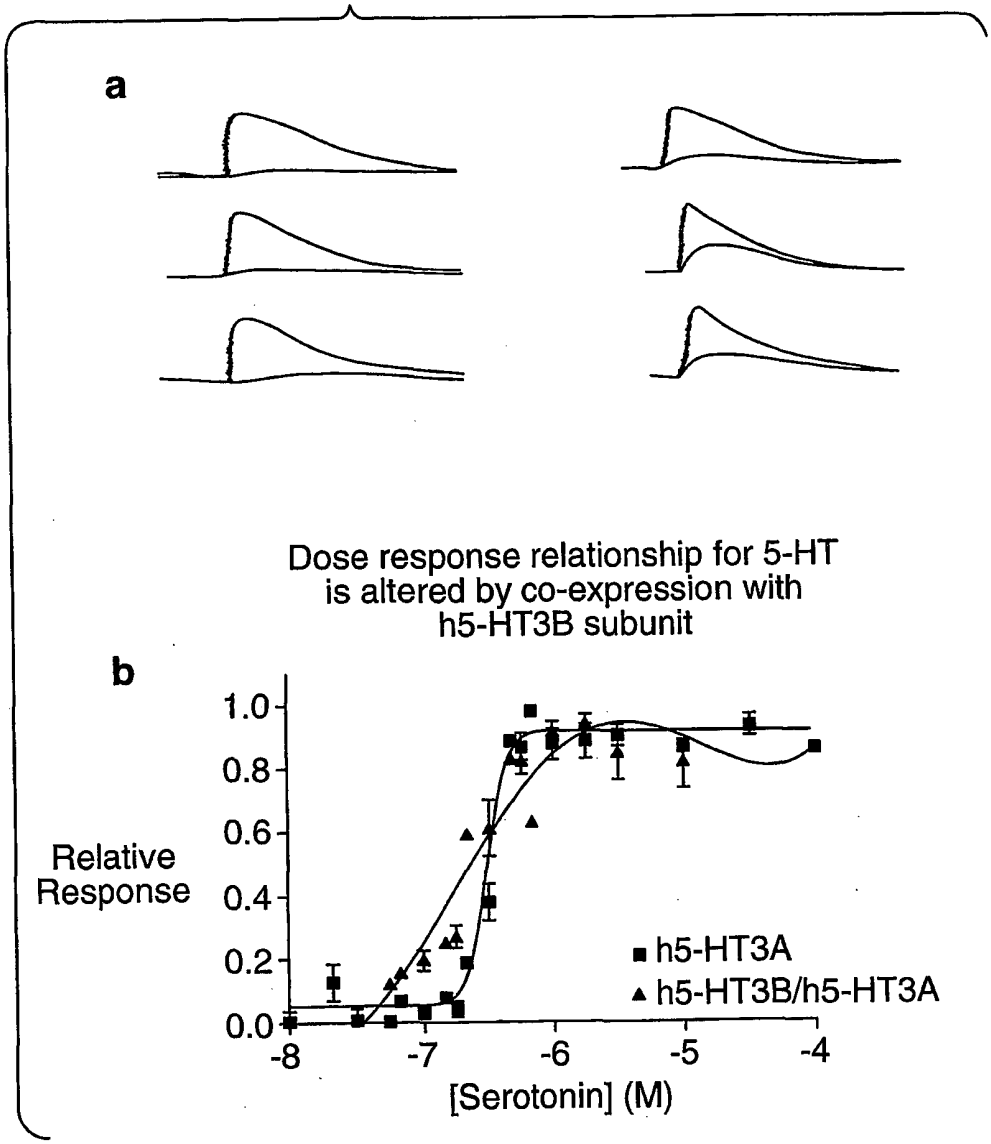
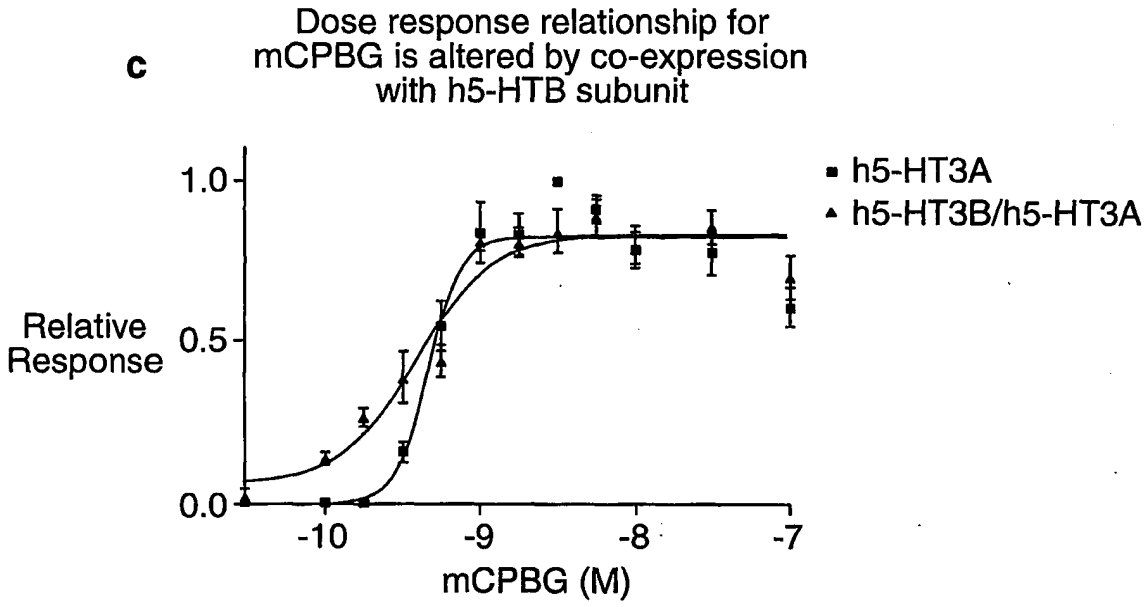

a
Dose response relationship for 5-HT
is altered by co-expression with
h5-HT3B subunit
b
Relative
Response
1.0
0.8
0.6
0.4
0.2
0.0
-8
-7
-6
-5
-4
[Serotonin] (M)
h5-HT3A
h5-HT3B/h5-HT3A

FIG. 9B


c
Dose response relationship for
mCPBG is altered by co-expression
with h5-HTB subunit
Relative
Response
1.0
0.5
0.0
-10
-9
-8
-7
mCPBG (M)
h5-HT3A
h5-HT3B/h5-HT3A

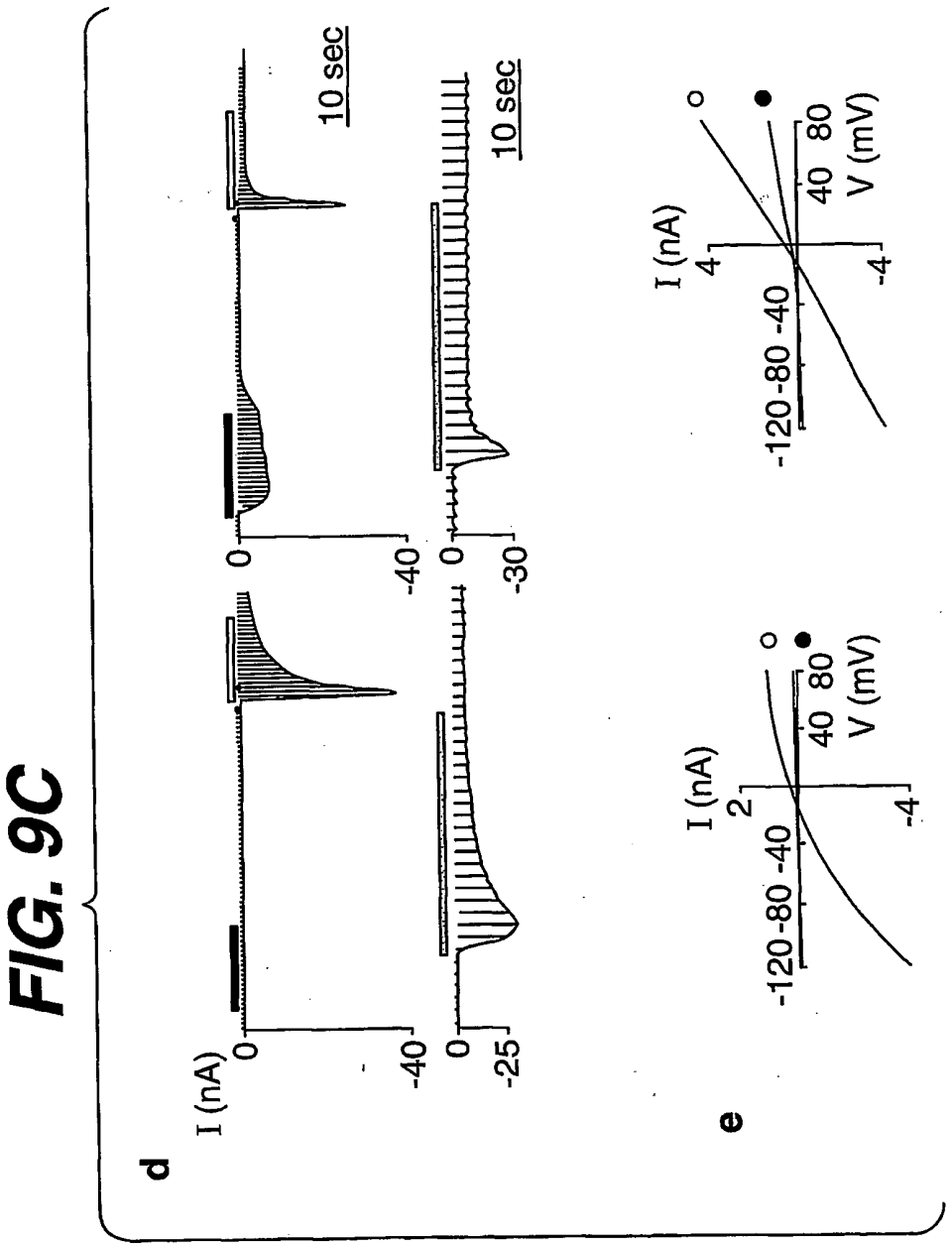

d
I (nA)
0
-40
0
-25
10 sec
10 sec
0
-40
0
-30
e
I (nA)
2
-120 -80 -40
40 80
V (mV)
-4
I (nA)
4
-120 -80 -40
40 80
V (mV)
-4

FIG. 9C

ORT1004

SEQUENCE LISTING

<110> Dubin, Adrienne
D'Andrea, Mike
Pyati, Jaushree
Zhu, Jessica Y
Erlander, Mark G

<120> DNA encoding a human subunit of the 5-ht3 serotonin receptor

<130> ORT-1004

<140>
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<160> 10

<170> PatentIn Ver. 2.0

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oligonucleotide

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<213> Artificial Sequence

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ORT1004

<223> Description of Artificial Sequence: BamHI primer

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<211> 40
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<213> Artificial Sequence

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ctggtggctg caggaattct agccacagat acacatcatc cccaggattc tgctctgtat 240
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gagaatcaaa tattaaagac aagtgtatgg taccaagagg tctggaatga tgaattttta 420
tcctggaact ccagcatgtt tgatgagatt agagagatct ccctacctct aagtgccatc 480
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tatgtttatg tgaactcatc tgggaccatt gagaactata agcccatcca ggtggtctct 600
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aagagcattc tgcatacagt ggaagacgta gacctggcct ttctgaggag cccagaagac 720
attcagcatg acaaaaaggc gtttttgaat gacagtgagt gggaacttct atctgtgtcc 780
tccacataca gcatcctgca gagcagcgct ggaggatttg cacagattca gtttaatgtg 840
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aag                                                               1923
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cagaaatatc ataaagaagt gagacctgtt tacaactgga ccaaggccac cacagtctac 180
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gatgagatta gagagatctc cctacctcta agtgccatct gggcccccga tatcatcatc 360
aatgagtttg tggacattga aagataccct gaccttccct atgtttatgt gaactcatct 420
gggaccattg agaactataa gcccatccag gtggtctctg cgtgcagttt agagacatat 480
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gaagacgtag acctggcctt tctgaggagc ccagaagaca ttcagcatga caaaaaggcg 600
tttttgaatg acagtgagtg ggaacttcta tctgtgtcct ccacatacag catcctgcag 660
agcagcgctg gaggatttgc acagattcag tttaatgtgg tgatgcgcag gcacccccctg 720
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ctgattgggc acttcttcac catctgcatg gccttcttgg ttctcagctt agctaagtcc 960
atcgtgttgg tcaaattcct ccatgatgag cagcgtggtg gacaggagca gcccttcttg 1020
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caggaggcag agtggctggt cctcctgtcc cgctttgacc gactgctctt ccaaagctac 1260
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gtgtga                                                            1326
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<210> 7
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<212> PRT
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  1               5                  10                  15

Ala Gly Ile Leu Ala Thr Asp Thr His His Pro Gln Asp Ser Ala Leu
             20                  25                  30

Tyr His Leu Ser Lys Gln Leu Leu Gln Lys Tyr His Lys Glu Val Arg
         35                  40                  45

Pro Val Tyr Asn Trp Thr Lys Ala Thr Thr Val Tyr Leu Asp Leu Phe
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     50                  55                  60

Val His Ala Ile Leu Asp Val Asp Ala Glu Asn Gln Ile Leu Lys Thr
 65                  70                  75                  80

Ser Val Trp Tyr Gln Glu Val Trp Asn Asp Glu Phe Leu Ser Trp Asn
                 85                  90                  95

Ser Ser Met Phe Asp Glu Ile Arg Glu Ile Ser Leu Pro Leu Ser Ala
            100                 105                 110

Ile Trp Ala Pro Asp Ile Ile Ile Asn Glu Phe Val Asp Ile Glu Arg
        115                 120                 125

Tyr Pro Asp Leu Pro Tyr Val Tyr Val Asn Ser Ser Gly Thr Ile Glu
    130                 135                 140

Asn Tyr Lys Pro Ile Gln Val Val Ser Ala Cys Ser Leu Glu Thr Tyr
145                 150                 155                 160

Ala Phe Pro Phe Asp Val Gln Asn Cys Ser Leu Thr Phe Lys Ser Ile
                165                 170                 175

Leu His Thr Val Glu Asp Val Asp Leu Ala Phe Leu Arg Ser Pro Glu
            180                 185                 190

Asp Ile Gln His Asp Lys Lys Ala Phe Leu Asn Asp Ser Glu Trp Glu
        195                 200                 205

Leu Leu Ser Val Ser Ser Thr Tyr Ser Ile Leu Gln Ser Ser Ala Gly
    210                 215                 220

Gly Phe Ala Gln Ile Gln Phe Asn Val Val Met Arg Arg His Pro Leu
225                 230                 235                 240

Val Tyr Val Val Ser Leu Leu Ile Pro Ser Ile Phe Leu Met Leu Val
                245                 250                 255

Asp Leu Gly Ser Phe Tyr Leu Pro Pro Asn Cys Arg Ala Arg Ile Val
            260                 265                 270

Phe Lys Thr Ser Val Leu Val Gly Tyr Thr Val Phe Arg Val Asn Met
        275                 280                 285

Ser Asn Gln Val Pro Arg Ser Val Gly Ser Thr Pro Leu Ile Gly His
    290                 295                 300

Phe Phe Thr Ile Cys Met Ala Phe Leu Val Leu Ser Leu Ala Lys Ser
305                 310                 315                 320

Ile Val Leu Val Lys Phe Leu His Asp Glu Gln Arg Gly Gly Gln Glu
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ORT1004

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                325                 330                 335

Gln Pro Phe Leu Cys Leu Arg Gly Asp Thr Asp Ala Asp Arg Pro Arg
            340                 345                 350

Val Glu Pro Arg Ala Gln Arg Ala Val Val Thr Glu Ser Ser Leu Tyr
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Gly Glu His Leu Ala Gln Pro Gly Thr Leu Lys Glu Val Trp Ser Gln
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Leu Gln Ser Ile Ser Asn Tyr Leu Gln Thr Gln Asp Gln Thr Asp Gln
385                 390                 395                 400

Gln Glu Ala Glu Trp Leu Val Leu Leu Ser Arg Phe Asp Arg Leu Leu
                405                 410                 415

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ORT1004

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/13071

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 1/00; C07H 21/04; C12N 1/20; C12P 21/06

US CL :530/350; 536/23.5; 435/252.3, 69.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.5; 435/252.3, 69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	DAVIES, P.A. et al., The 5-HT3b subunit is a major determinant of serotonin-receptor function. Leters to Nature. 28 January 1999, Vol. 397, pages 359-363, especially page 360 and attached sequence alignment.	1-11, 14-23 --- 12, 13
Y	US 5,766,879 A (GERALD et al.) 16 June 1998, especially col. 13.	12, 13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 DECEMBER 2000	Date of mailing of the international search report 03 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Michael Brannock Telephone No. (703) 308-0196 DELLA MAE COLLINS PARALEGAL SPECIALIST TECHNOLOGY CENTER 1600

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/13071

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (*Continuation of item 2 of first sheet*)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

COMMERCIAL NUCLEIC ACID AND PROTEIN DATABASES
STN: MEDLINE, BIOSIS, BIOTECHNO
WEST 2.0
Search Terms: setotonin, 5-HT, receptor, clone, genomic

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-11 and 14, drawn to polynucleotides, polypeptides, vectors, host cells and methods of producing a polypeptide.
Group II, claim(s) 12-13, drawn to antibodies.
Group III, claim(s) 15-18, drawn to methods of identifying modulators of a polypeptide.
Group IV, claim(s)19-22, drawn to agonists and antagonists of a polypeptide.
Group V, claim(s) 23, drawn to a method of treatment.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under *PCT Rule 13.2, they lack the same or corresponding special technical features* for the following reasons: Pursuant to 37 C.F.R. 1.475(d), this Authority considers that the main invention in the instant application comprises the first recited product, a polynucleotide of SEQ ID NO: 1 and 2, and the first recited method of using that product, namely in the process of producing the encoded polypeptide of SEQ ID NO: 3 and. However, the examiner has noted that the SEQ ID NOs recited in the claims match the figure numbers and not those of the SEQ ID NOs provided in the sequence listing. It is assumed that the claims were intended to be directed to polynucleotides of SEQ ID NO: 4 and 5 encoding a polypeptide of SEQ ID NO: 7. Therefore, this authority considers the that the main invention in the instant application comprises the first recited product, a polynucleotide of SEQ ID NO: 4 and 5, and the first recited method of using that product, namely in the process of producing the encoded polypeptide of SEQ ID NO: 7. Note that there is no method of making the polynucleotide. Also included in this group is the product made, namely the encoded polypeptide of SEQ ID NO: 7, and vectors and host cells comprising the polynucleotide and expressed polypeptide. Further, pursuant to 37 C.F.R. 1.475(b)-(d), the ISA/US considers that the materially and functionally dissimilar products of Groups II and IV and the additional methods of Groups III and V do not correspond to the main invention. This Authority therefore considers that the several inventions do not share a special technical feature within the meaning of PCT Rule 13.2 and thus do not relate to a single general inventive concept within the meaning of PCT Rule 13.1.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
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30 November 2000 (30.11.2000)

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(10) International Publication Number
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(51) International Patent Classification[7]: C07K 1/00, C07H 21/04, C12N 1/20, C12P 21/06

(21) International Application Number: PCT/US00/13071

(22) International Filing Date: 11 May 2000 (11.05.2000)

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(30) Priority Data:
09/317,713 24 May 1999 (24.05.1999) US

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

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Published:
— *with international search report*

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WO 00/71741 A3

(54) Title: DNA ENCODING A HUMAN SUBUNIT OF THE 5-HT3 SEROTONIN RECEPTOR

(57) Abstract: DNA encoding human 5-HT3-B has been cloned and characterized. The recombinant protein is capable of forming biologically active human 5-HT3-B protein. The cDNA has been expressed in recombinant host cells that produce active recombinant protein. In addition, the recombinant host cells are utilized to establish a method for identifying modulators of the receptor activity, and receptor modulators are identified.

TITLE OF THE INVENTION

DNA ENCODING A HUMAN SUBUNIT OF THE 5-HT3 SEROTONIN RECEPTOR

BACKGROUND OF THE INVENTION

Serotonin (5-hydroxytryptamine, 5-HT) is a multifunctional chemical transmitter that signals though cell surface receptors. At least fourteen subtypes of serotonin receptors have been defined pharmacologically (Julius, 1996; Tecott and Julius, 1993). Thirteen of the fourteen known receptors are G-protein coupled receptors and the only known ionotropic 5-HT receptor, the type 3 5-HT3 receptor, is a fast activating, ligand gated non-selective cation channel unique among known monoamine receptors (Derkach et al., 1989). The 5-HT3 receptor is exclusively localized on neurons in the central (Waeber et al., 1989; Yakel et al., 1991) and peripheral (Fozard, 1984) nervous systems. Activation of the 5-HT3 receptor leads to membrane depolarization and an increase in intracellular Ca^{2+}. The 5-HT3 receptor is the target of antagonists (granisetron and ondansetron) selective against the nausea induced by cytotoxic chemotherapy and general anesthesia (Gralla, 1998). Evidence is accumulating that serotonin 5-HT3 receptors are important in pain reception, anxiety, cognition, cranial motor neuron activity, sensory processing, modulation of affect, and the behavioral consequences of drug abuse (Lambert et al., 1995; Passani and Corradetti, 1996).

The 5-HT3 receptor is thought to be a homopentimeric protein with multiple agonist and allosteric ligand binding sites (Boess et al., 1995; Bonhaus et al., 1995; Eglen and Bonhaus, 1996; Green et al., 1995; Hargreaves et al., 1996; Lambert et al., 1995; Van Hooft et al., 1997; Wetzel et al., 1998). The full coding sequence of the 5-HT3 receptor has been cloned from mouse (Hope et al., 1993; Maricq et al., 1991; Werner et al., 1994), rat (Miyake et al., 1995), guinea pig (Lankiewicz et al., 1998), and human (Belelli et al., 1995; Miyake et al., 1995); AJ003079 Bruss et al., unpublished). It has structural and functional similarities with nicotinic, GABA-

ergic and other ligand gated ion channels (Barnard, 1996; Gurley and Lanthorn, 1998; Maricq et al., 1991). Like other receptors of the same ligand-gated cation channel superfamily (Changeux and Edelstein, 1998; Lena and Changeux, 1993), the 5-HT3 receptors rapidly desensitize (Peters and Lambert, 1989; Yakel et al., 1991). The pharmacological and kinetic profile of the members of this superfamily depends on subunit composition (Chang et al., 1995; Harris et al., 1995; Lindstrom et al., 1990; Luetje and Patrick, 1991; Olsen, 1998).

While it is thought that only one gene encoding the 5-HT3 receptor exists (5-HT3-A receptor), several lines of evidence indicate that 5-HT3 receptors may exist as heteromultimers. First, receptors purified from a variety of sources by affinity chromatography usually reveal at least 2 major protein bands with molecular masses in the order of 54 and 38 kDa (Lambert et al., 1995). The 5-HT3-A receptor corresponds to the former (Turton et al., 1993). Affinity purified 5-HT3 receptor solubilized from pig cerebral cortex is composed of at least 3 separable components, based on silver staining of proteins on denaturing gels (Fletcher and Barnes, 1997). A number of these protein bands are not recognized by specific antibodies directed against the recombinant 5-HT3-A subunit (Fletcher and Barnes, 1997), and their sizes are too large (52-71 kDa) to be considered as degraded 5-HT3-A fragments (Fletcher and Barnes, 1998).

Second, expression of the recombinant receptor in Xenopus oocytes or mammalian cell lines often do not reveal all the electrophysiological and pharmacological properties of the native receptor (Gill et al., 1995; Lambert et al., 1995; Van Hooft et al., 1997). Differences in desensitization kinetics, single channel conductance and agonist efficacy have been observed and may be due to the lack of an endogenous subunit not present in the recombinant cell lines or oocyte system. Two forms of the receptor subunit with about 98% identity have been observed in mouse, rat, guinea pig and human. The two forms differ by the insertion of 6 to 32 consecutive amino acids and may be produced by alternative splicing of a single gene

(Uetz et al., 1994; Werner et al., 1994). While most of the pharmacological and electrophysiological characteristics of the recombinant mouse isoforms are similar (Downie et al., 1994; Niemeyer and Lummis, 1998; Werner et al., 1994), the efficacy of 2-methyl-5-HT and/or m-chlorophenylbiguanide (mCPBG) differ (Downie et al., 1994; Niemeyer and Lummis, 1998; Van Hooft et al., 1997). The two variants may coexist in the same cell lines and heteromultimers containing both forms may explain some but not all of the differences observed between homomultimers of recombinant subunits and the native cells.

Third, 5-HT-3 receptors in different preparations from the same species reveal variation in electrophysiological and pharmacological properties (Fletcher and Barnes, 1998; Richardson and Engel, 1986). In particular, there appears to be heterogeneity of desensitization kinetics across cells, and even within the same cell type (Lambert et al., 1995). Differences in desensitization kinetics have been observed in NG108-15 cells under various differentiation states (Shao et al., 1991). While this difference may be due to heterogeneity of subunits, it may also be a consequence of different post-translational states of the receptor. For instance, the rate of desensitization of nicotinic acetylcholine and $GABA_A$ receptors is enhanced by phosphorylation (Raymond, 1998; Raymond et al., 1993; Swope et al., 1992). A wide range of single channel conductance values has been reported for the 5-HT3 receptor (Fletcher and Barnes, 1998), however, this difference may be due to the phosphorylation state of the receptor in different cells. Van Hooft and colleagues have shown that phosphorylation controls the conductance of 5-HT-3 receptors in N1E-115 cells (Van Hooft and Vijverberg, 1995). Furthermore, significant differences in rectification properties of the channel have been reported in different cell types (Hussy et al, 1994).

Fourth, differentiated murine N1E-115 cells express native 5-HT-3 receptors with an efficacy for 2-methyl-5-HT higher than that observed for either murine

recombinant 5-HT3-A isoform expressed in oocytes separately or together. However, co-expression of recombinant 5-HT3-A receptor in oocytes with mRNA isolated from differentiated murine N1E-115 cells reconstitutes the functional properties of the native receptor expressed on differentiated cells (Van Hooft et al., 1997).

While it has been reported that the 5-HT3 receptor can co-assemble with the nicotinic alpha4 subunit in Xenopus oocyte expression studies (Van Hooft et al., 1998), nicotinic ACh receptor subunits were not associated with native porcine brain 5-HT3 receptors (Fletcher et al., 1998).

Fifth, allosteric modulation of the receptor by Zn^{2+} has different effects on the recombinant murine 5-HT3-A receptor expressed in oocytes (enhancement) compared to its effects on 5-HT receptor-mediated currents in NCB20 cells (block; (Lovinger, 1991), the cell line from which the 5-HT3-A receptor used in these studies was cloned. The sensitivity of another member of this superfamily of ligand-gated receptors, $GABA_A$ receptors, to blockade by zinc ions is known to depend on subunit composition (Smart et al., 1994).

Recently the sequence of human 5-HT3-B was disclosed and shown to alter functional characteristics of 5-HT3-A (Davies et al 1999). The functional characteristics described for human 5-HT3-B by Davies et al. (1999) included a decreased affinity for 5-HT with no effect on the affinity for other agonists including mCPBG. This art did not find that the affinity and cooperativity for 5-HT3 receptor function were increased and decreased, respectively, by 5-HT3-B, nor did it report any affects on current kinetics.

The isolation and functional characterization of a human cDNA encoding a modifier subunit for the serotonin 5-HT3 receptor explains observations that 5-HT3 receptors from a variety of preparations have distinct pharmacological,

kinetic and voltage-dependent properties (Peters et al., 1992). Expression of this human 5-HT3 subunit, termed 5-HT3-B will further aid in discovery of serotonin function and can be used to screen for compounds that modulate function of a heteromeric receptor complex to which the 5-HT3-B receptor participates.

5

SUMMARY OF THE INVENTION

A DNA molecule encoding a human subunit with homology to 5-HT3-A serotonin receptor that, when co-expressed with the short form of the serotonin 5-HT3-A receptor, modifies the functional and pharmacological characteristics of
10 the 5-HT3 receptor has been cloned and characterized. Using a recombinant expression system, functional DNA molecules encoding the human serotonin 5-HT3 receptor modifier protein (heretofore designated 5-HT3-B) have been isolated. The biological and structural properties of these proteins are disclosed, as is the amino acid and nucleotide sequence. The recombinant protein is useful
15 to identify modulators of human 5-HT3 receptors composed of both 5-HT3 receptors and the modifier subunit (5-HT3-B). Modulators identified in the assay disclosed herein are useful as therapeutic agents, include, but are not limited to, nausea, depression, anxiety, psychoses (for example schizophrenia), urinary continence, Huntington's chorea, tardive dyskinesia, Parkinson's disease, obesity,
20 hypertension, migraine, Gilles de la Tourette's syndrome, sexual dysfunction, drug addiction, drug abuse, cognitive disorders, learning, Alzheimer's disease, cerebral coma, senile dementia, obsessive-compulsive behavior, panic attacks, pain, social phobias, eating disorders and anorexia, cardiovascular and cerebrovascular disorders, non-insulin dependent diabetes mellitus, hyperglycemia, constipation,
25 arrhythmia, disorders of the neuroendocrine system, stress, and spasticity, as well as acid secretin, ulcers, airway constriction, asthma, allergy, inflammation, and prostate dysfunction, and diagnostic agents. The recombinant DNA molecules and portions thereof, are useful for isolating homologues of the DNA molecules, identifying and isolating genomic equivalents of the DNA molecules, and
30 identifying, detecting or isolating mutant forms of the DNA molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 – [SEQ.ID.NO.:5] Nucleic Acid sequence of the human 5-HT3-B (full sequence including untranslated regions) is shown;1923 bases (141 bases of 5' UTR; 456 bases of 3' UTR).

Figure 2 - [SEQ.ID.NO.:6] Nucleotide sequence for the coding region of 5-HT3-B is shown; 1326 bases.

Figure 3 - [SEQ.ID.NO.:7] The amino acid sequence of human 5-HT3-B is shown (441 amino acids).

Figure 4 – PANEL A [Figure 4A]. The PCR-based tissue distribution of the human 5-HT3-B is shown. Lanes are labeled as indicated: 1, Cerebellum; 2, Cerebral cortex; 3, Medulla; 4, Spinal Cord; 5, Occipital pole; 6, frontal lobe; 7, Temporal lobe; 8, Putamen; 9, Amygdala; 10, Caudate nucleus; 11, Corpus callosum; 12, Hippocampus; 13, Whole brain; 14, Substantia nigra; 15, Thalamus; 16, Heart; 17, Brain; 18, Placenta; 19, Lung; 20, Liver; 21, Skeletal muscle; 22, Kidney; 23, Pancreas; 24, Spleen; 25, Thymus; 26, Prostate; 27, Testis; 28, Ovary; 29, Small Intestine; 30, Colon (mucosal lining); 31, Peripheral blood leukocyte.

PANEL B [Figure 4B]. RT-PCR in situ hybridization was performed on normal human tissues of the spleen (b1, b2) and small intestine (b3, b4). Human spleen had intense 5-HT3-B mRNA (b1) and 5HT3-A mRNA (b2) signal (purple in color – dark grey in black-and-white), respectively, in small lymphocytes (arrows). Similarly, intense 5-HT3-B mRNA (b3) and 5HT3-A mRNA (b4) signal (purple in color – dark grey in black-and-white) was detected in stromal fibroblasts of the small intestines (arrows). Total magnification = 600x.

PANEL C [Figure 4C]. RT-PCR in situ hybridization was performed on serial 10 μm sections of the normal monkey amygdala. Figures 4c1 (300x) and 4c3 (600x) show positive 5-HT3-A mRNA signal (purple in color – dark grey in black-and-white) in seven neurons (arrows) which is also show positive 5HT3-B mRNA signal (4c2 (300x); 4c4 (600x)). Not all cells were labeled by the RT-PCR ISH technique using specific probes for 5-HT3-B or 5-HT3-A.

Figure 5 - PANEL A [Figures 5A and 5B]. Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: 5-HT3-B modifies the kinetics and magnitude of 5-HT3-A currents elicited by a subset of agonists. (a.) 5-HT3-B normalizes agonist-induced 5-HT3-A responses in *Xenopus* oocytes. 5-HT3-A-expressing oocytes responded to 5-HT, 2-methyl-5-HT, mCPBG and 1-PBG with either a slowly desensitizing response (left panel; "slow" responders, 71% of oocyte batches), or a complex current that included a rapidly desensitizing component (right panel; "fast" responders). Oocytes expressing both subunits produced uniform responses (middle panel). The agonists tested included 5-HT (0.3 and 10 μM, responses are superimposed); mCPBG (0.3 and 10 μM); 1-PBG (10 and 100 μM); DA (0.1 and 1 mM); 2-Me-5-HT (10 μM). Agonists are applied during the time indicated by the horizontal bar above the record. The clear bar extending from the solid bar indicates the longer exposure to agonist for one of the superimposed traces. Oocytes were continuously perfused with Ba^{2+} containing saline at a rate of 10 ml/min at room temperature. Time scale bar: 40 sec. (b.) Responses to 1-PBG were blocked by the 5-HT3 antagonist tropisetron. After obtaining a control response to 1-PGB (100 μM; indicated by the solid bar) (left panel), the oocyte was washed 2 min then incubated for 30 sec in tropisetron (1 μM; clear bar) then challenged with both 1-PBG and tropisetron. The response to 1-PBG was completely blocked (middle panel). The response to agonist recovered after a 2 min washout of antagonist (right panel).

PANEL B [Figure 5C]. Peak currents elicited by the indicated agonists from oocytes expressing 5-HT3-A (grey bars) and both 5-HT3-B and 5-HT3-A (solid bars). Data from oocytes recorded in Ba^{2+}- and Ca^{2+}- containing saline were combined since there were no differences in 5-HT (10 μM)-induced maximal current in Ca^{2+}-containing vs. Ba^{2+}-containing saline (-7.7 +/- 1.4 μA (n=16) vs. -7.6 +/- 0.9 μA (n=24)). The number of oocytes tested ranged from 9 to 61; SEM values were 10% for 5-HT (10 μM) and 21% for both mCPBG (10 μM) and 1-PBG (100 μM). p values are indicated: * $p<0.05$, ** $p<0.01$ (Students t-test).

Figure 6 - Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: 5-HT3-B normalized the 5-HT response t80 and time to peak to similar values in "slow" (a,c) and "fast" (b,d) responders. (a.) t80 of responses elicited by 5-HT (concentrations indicated) from experiments in which 5-HT3-A-injected oocytes respond to 5-HT with a slowly decaying response. 5-HT3-A-injected oocytes (grey bars; n were 8, 38 and 13 individual oocytes, respectively); 5-HT3-A receptor and 5-HT3-B -injected oocytes (solid bars; n were 11, 33 and 16 individual oocytes, respectively). The differences were significant at all agonist concentrations ($p< 0.05$, $5e^{-9}$, 0.005 for 1, 10 and 100 μM 5-HT, respectively). (b.) t80 of responses elicited by 5-HT from experiments in which 5-HT3-A-injected oocytes respond to 5-HT with a rapidly decaying response. 5-HT3-A injected oocytes (grey bars; n were 5, 11 and 10 individual oocytes, respectively); 5-HT3-A receptor and 5-HT3-B injected oocytes (solid bars; n were 9, 12 and 3 individual oocytes, respectively). Significant differences were observed at 1 and 10 μM 5-HT ($p < 0.005$ and $p< 5e^{-5}$, respectively). (c.) The time to peak of the response to 10 μM 5-HT in slow responders is significantly faster when 5-HT3-B is co-expressed with 5-HT3-A (solid bars, n= 26) compared to 5-HT3-A alone (grey bars, n=41, $p<0.0005$). (d.) The time to peak of the response to 10 μM 5-HT in "fast" responders was significantly slower

when 5-HT3-B is co-expressed (solid bars, n=12) compared to homomers (grey bars, n=10, $p<0.05$).

Figure 7 - Functional expression of human 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes: Agonist dose response relationships are altered in the presence of 5-HT3-B and depend on the ratio of 5-HT3-B to 5HT3-A cRNA injected in Xenopus oocytes. (a.) Oocytes were injected with either 5-HT3-A or both 5-HT3-A and 5-HT3-B cRNA and tested for their response to the indicated concentrations of 5-HT. The data are presented relative to the maximum response elicited by 100 μM 5-HT in the same oocytes. 5-HT3-B decreases the apparent affinity of the 5HT3-A receptor for 5-HT. The agonist was applied to the cell at a rate of 10 ml per min in bath perfusate. The data were analyzed using GraphPad Prizm and fit with a Boltzmann exponential. (b.) The percent maximum response for 0.3 μM 5-HT, 0.3 μM mCPBG and 10 μM 1-PBG are plotted for oocytes injected with 5-HT3-A alone (left-most stippled bars), both subunits at a 1:10 ratio (5-HT3-A to 5-HT3-B, solid bars), both subunits at a 1:1 ratio (hatched bars), both subunits at a 1:0.1 ratio (clear bars), and both subunitis at a 1: 0.01 ratio (cross-hatched bars). The percent of the maximal response obtained in individual oocytes was averaged. Significant differences are indicated by asterisks (* $p<0.05$; ** $p< 0.01$).

Figure 8- Specificity of the modulatory effect of human 5-HT3-B in oocytes is shown. In all cases the amount of injected 5-HT3-B cRNA was at least 2-fold the concentration of each nACh receptor. (a.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha3\beta4$ with (solid; n= 5) and without (grey; n=4) 5-HT3-B. (b.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha4\beta2$ with (solid; n=8) and without (grey; n=9) 5-HT3-B. (c.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor $\alpha2\beta2$ with (solid; n=3) and without (grey; n=3) 5-HT3-B.

(d.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α7 with (solid; n=12) and without (grey; n=15) 5-HT3-B.

Figure 9 - PANEL A [Figures 9A and 9B]. Functional expression of human 5-HT3-B in recombinant host cells is shown: 5-HT3-B and 5-HT3-A heteromers display pharmacological and voltage-dependent properties distinct from 5-HT3-A homomeric receptors. (a.) Ca^{2+} influx induced by agonist challenge was determined using the Ca^{2+} sensitive dye Fluo-4 on the FLIPR system. Responses to 0.17 and 3 μM 5-HT (top), 6 and 30 μM 1-PBG (middle), 0.3 and 3 μM mCPBG are shown. Each record represents 3.33 min. Agonists were added after 20 seconds and were present throughout the recording. Left: 5-HT3-A/HEK cells; Right: 5-HT3-A/5-HT3-B/HEK cells. (b.) Dose response for 5-HT - activated Ca^{2+} influx using the FLIPR system. 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) had significantly higher affinity toward 5-HT and decreased nH compared to 5-HT3-A homomeric receptors (squares). Peak responses were determined and normalized to the maximum observed response. Mean values from 4-10 separate experiments are presented. Data were fit with Boltzmann exponentials.

PANEL B [Figure 9C]. Dose response for mCPBG-activated Ca^{2+} influx using the FLIPR system. Data from 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) and 5-HT3-A homomeric receptors (squares) is shown. Peak responses were determined and normalized to the maximum observed response. Values were averaged from 4 separate experiments. Data were fit with Boltzmann exponentials.

PANEL C [Figures 9D and 9E]. Voltage clamp recordings from 5-HT3-A/HEK cells (left) and 5-HT3-A/5-HT3-B/HEK cells (right). Spikes in the current record are the currents induced by voltage ramp protocols used to determine the change in whole cell membrane conductance. Voltage ramps were

evoked every second. (d.) Top: inward currents elicited by 10 μM 1-PBG (solid bar) and 10 μM 5-HT (clear bar) from a holding potential of -68 mV. Bottom: inward currents elicited by 100 μM 1-PBG (hatched bar). (e.) The voltage relationship for the agonist induced currents obtained using a voltage ramp protocol. 5-HT induced currents from transfected HEK293 cells expressing the homomeric receptor (left) and 5-HT3-A and 5-HT3-B subunits (right). A voltage ramp was applied to the cell from -120 to +80 mV (not corrected for a junction potential of -18 mV) over 200 msec (1 mV/ms) before (solid circle) and during (clear circle) agonist application. The V_{rev} were similar for both responses and were near 0 mV. Asterisks above the recordings in (d) indicate the ramp currents shown on an expanded scale in (e).

DETAILED DESCRIPTION

The present invention relates to DNA encoding human 5-HT3-B, which was isolated from a cDNA library from human small intestine. The human 5-HT3-B, as used herein, refers to protein, which can specifically function as a receptor in a complex with the 5-HT3-A receptor.

The complete amino acid sequence of the human 5-HT3-B was not known, nor was the complete nucleotide sequence encoding human 5-HT3-B known prior to the cloning and functional determination of the present invention. However, the cloning of a full length DNA molecule encoding human 5-HT3-B and some aspects of the function of the protein encoded by this molecule was recently reported (Davies et al., 1999).

The present invention provides a human 5-HT serotonin receptor complex that has distinct pharmacological, kinetic and voltage-dependent properties, which mimic native responses to a greater degree than previously described. Thus the invention described herein shows that 5-HT3-B confers distinct pharmacological, kinetic and voltage-dependent properties upon the 5-HT3-A receptor. The

present invention shows that 5-HT3-B specifically interacts with the 5-HT3-A and not the nicotinic ACh receptors α3β4, α2β2, α4β2 and α7. Furthermore, the mRNA encoding the invention described herein is co-localized within neurons in the monkey amygdala and human cerebral cortex. The present invention further shows that 5-HT3-B and 5-HT3-A mRNA are expressed in lymphocytes and epithelial cells of peripheral organs including the spleen and small intestine. The physiological significance of the novel findings reported herein include the ability of cells co-expressing 5-HT3-B and 5-HT3-A to be more sensitive to 5-HT than cells expressing single receptors, and to have a altered response duration to agonist. These alterations in receptor-mediated current could have profound effects on 5-HT activation of neuronal excitability. It is predicted that a wide variety of cells and cell types will contain the human 5-HT3-B. Vertebrate cells capable of producing human 5-HT3-B include, but are not limited to human 5-HT3-B -expressing cells isolated from cells that show sensitivity to or bind serotonin.

Other cells and cell lines may also be suitable for use to isolate human 5-HT3-B cDNA. Selection of suitable cells may be done by screening for the response to 1-phenylbiguanide (1-PBG) or mCPBG, either the magnitude of the response at low micromolar concentrations of 1-PBG or mCPBG, or the rate of decay of the cellular response elicited by 1-PBG, mCPBG, or serotonin. Human 5-HT3-B activity can be monitored by performing a ^{3}H- [mCPBG] binding assay in the presence of 5-HT3-A receptor (Steward et al., 1993), by direct measurement of a Ca^{+2} influx using the Ca^{+2} sensitive dyes (Kuntzweiler et al., 1998), or by net ion flux using voltage clamp techniques (Hamill et al., 1981). Cells that possess isolate human 5-HT3-B activity in this assay may be suitable for the isolation of isolate human 5-HT3-B DNA or mRNA.

Any of a variety of procedures known in the art may be used to molecularly clone human 5-HT3-B DNA. These methods include, but are not

limited to, direct functional expression of the human 5-HT3-B genes following the construction of a human 5-HT3-B-containing cDNA library in an appropriate expression vector system. Another method is to screen human 5-HT3-B -containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a labelled oligonucleotide probe designed from the amino acid sequence of the human 5-HT3-B subunits. An additional method consists of screening a human 5-HT3-B -containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a partial cDNA encoding the human 5-HT3-B protein. This partial cDNA is obtained by the specific PCR amplification of human 5-HT3-B DNA fragments through the design of degenerate oligonucleotide primers from the amino acid sequence of the purified human 5-HT3-B protein.

Another method is to isolate RNA from human 5-HT3-B -producing cells and translate the RNA into protein via an *in vitro* or an *in vivo* translation system. Translation of the RNA into a peptide a protein will result in the production of at least a portion of the human 5-HT3-B protein which an be identified by, for example, immunological reactivity with an anti- human 5-HT3-B antibody or by biological activity of human 5-HT3-B protein. In this method, pools of RNA isolated from human 5-HT3-B -producing cells can be analyzed for the presence of an RNA that encodes at least a portion of the human 5-HT3-B protein. Further fractionation of the RNA pool can be done to purify the human 5-HT3-B RNA from non- human 5-HT3-B RNA. The peptide or protein produced by this method may be analyzed to provide amino acid sequences which in turn are used to provide primers for production of human 5-HT3-B cDNA, or the RNA used for translation can be analyzed to provide nucleotide sequences encoding human 5-HT3-B and produce probes for this production of human 5-HT3-B cDNA. This method is known in the art and can be found in, for example, Maniatis, T., Fritsch, E.F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989.

It is readily apparent to those skilled in the art that other types of libraries, as well as libraries constructed from other cells or cell types, may be useful for isolating human 5-HT3-B -encoding DNA. Other types of libraries include, but are not limited to, cDNA libraries derived from other cells, from organisms other than human, and genomic DNA libraries that include YAC (yeast artificial chromosome) and cosmid libraries.

It is readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from cells or cell lines which have human 5-HT3-B activity. The selection of cells or cell lines for use in preparing a cDNA library to isolate human 5-HT3-B cDNA may be done by first measuring cell-associated human 5-HT3-B activity using the measurement of human 5-HT3-B -associated biological activity or a human 5-HT3-B - 5-HT3-A receptor ligand binding assay [^{3}H-mCPBG] (Steward et al., 1993).

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Maniatis, T., Fritsch, E.F., Sambrook, J., Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

It is also readily apparent to those skilled in the art that DNA encoding human 5-HT3-B may also be isolated from a suitable genomic DNA library. Construction of genomic DNA libraries can be performed by standard techniques well known in the art. Well known genomic DNA library construction techniques can be found in Maniatis, T., Fritsch, E.F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

In order to clone the human 5-HT3-B gene by the above methods, the amino acid sequence of human 5-HT3-B may be necessary. To accomplish this, human 5-HT3-B protein may be purified and partial amino acid sequence determined by automated sequencers. It is not necessary to determine the entire amino acid sequence, but the linear sequence of two regions of 6 to 8 amino acids from the protein is determined for the production of primers for PCR amplification of a partial human 5-HT3-B DNA fragment.

Once suitable amino acid sequences have been identified, the DNA sequences capable of encoding them are synthesized. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human 5-HT3-B sequence but will be capable of hybridizing to human 5-HT3-B DNA even in the presence of DNA oligonucleotides with mismatches. The mismatched DNA oligonucleotides may still sufficiently hybridize to the human 5-HT3-B DNA to permit identification and isolation of human 5-HT3-B encoding DNA. DNA isolated by these methods can be used to screen DNA libraries from a variety of cell types, from invertebrate and vertebrate sources, and to isolate homologous genes.

Purified biologically active human 5-HT3-B may have several different physical forms. Human 5-HT3-B may exist as a full-length nascent or unprocessed polypeptide, or as partially processed polypeptides or combinations of processed polypeptides. The full-length nascent human 5-HT3-B polypeptide may be posttranslationally modified by specific proteolytic cleavage events that result in the formation of fragments of the full-length nascent polypeptide. A fragment, or physical association of fragments may have the full biological activity associated with human 5-HT3-B however, the degree of human 5-HT3-B

activity may vary between individual human 5-HT3-B fragments and physically associated human 5-HT3-B polypeptide fragments.

The cloned human 5-HT3-B DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant human 5-HT3-B protein. Techniques for such manipulations are fully described in Maniatis, T, et al., supra, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria including E. coli, blue-green algae, plant cells, insect cells, fungal cells including yeast cells, and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungal cells or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one that causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant human 5-HT3-B in mammalian cells. Commercially available

mammalian expression vectors which may be suitable for recombinant human 5-HT3-B expression, include but are not limited to, pMAMneo (Clontech), pcDNA3 (InVitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1 (8-2) (ATCC 37110), pdBPV-MMTneo (342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lZD35 (ATCC 37565).

A variety of bacterial expression vectors may be used to express recombinant human 5-HT3-B in bacterial cells. Commercially available bacterial expression vectors that may be suitable for recombinant human 5-HT3-B expression include, but are not limited to pET vectors (Novagen) and pQE vectors (Qiagen).

A variety of fungal cell expression vectors may be used to express recombinant human 5-HT3-B in fungal cells such as yeast. Commercially available fungal cell expression vectors which may be suitable for recombinant human 5-HT3-B expression include but are not limited to pYES2 (InVitrogen) and Pichia expression vector (InVitrogen).

A variety of insect cell expression vectors may be used to express recombinant human 5-HT3-B in insect cells. Commercially available insect cell expression vectors that may be suitable for recombinant expression of human 5-HT3-B include but are not limited to pBlueBacII (InVitrogen).

DNA encoding human 5-HT3-B may be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as E. coli, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to drosophila and silkworm derived cell lines. Cell lines derived

from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), L-cells, and HEK-293 (ATCC CRL1573).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, protoplast fusion, lipofection, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce human 5-HT3-B protein. Identification of human 5-HT3-B expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti- human 5-HT3-B antibodies, and the presence of host cell-associated human 5-HT3-B activity.

Expression of human 5-HT3-B DNA may also be performed using *in vitro* produced synthetic mRNA. Synthetic mRNA or mRNA isolated from human 5-HT3-B producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being generally preferred.

To determine the human 5-HT3-B DNA sequence(s) that yields optimal levels of human 5-HT3-B activity and/or human 5-HT3-B protein, human 5-HT3-B DNA molecules including, but not limited to, the following can be constructed: the full-length open reading frame of the human 5-HT3-B cDNA encoding the 47.9 kDa protein from approximately base 142 to approximately base 1465 (these numbers correspond to first nucleotide of first methionine and last

nucleotide before the first stop codon) and several constructs containing portions of the cDNA encoding human 5-HT3-B protein. All constructs can be designed to contain none, all or portions of the 5' or the 3' untranslated region of human 5-HT3-B cDNA. Human 5-HT3-B activity and levels of protein expression may be determined following the introduction, both in combination with 5-HT3-A or alone, of these constructs into appropriate host cells. Following determination of the human 5-HT3-B DNA cassette yielding optimal expression in transient assays, this human 5-HT3-B DNA construct is transferred to a variety of expression vectors, for expression in host cells including, but not limited to, mammalian cells, baculovirus-infected insect cells, E. coli, and the yeast S. cerevisiae.

Host cell transfectants and microinjected oocytes may be used to assay both the levels of human 5-HT3-B activity and levels of human 5-HT3-B protein by the following methods. In the case of recombinant host cells, this involves the co-transfection of one or possibly two or more plasmids, containing the human 5-HT3-B DNA encoding one or more fragments or subunits and the 5-HT3-A receptor or transfection of the human 5-HT3-B protein into human cell lines expressing the 5-HT3-A receptor. In the case of oocytes, this involves the co-injection of synthetic RNAs for human 5-HT3-B and 5-HT3-A receptor proteins. Following an appropriate period of time to allow for expression, cellular protein is metabolically labelled with, for example ^{35}S-methionine for 24 hours, after which cell lysates and cell culture supernatants are harvested and subjected to immunoprecipitation with polyclonal antibodies directed against the human 5-HT3-B protein.

Other methods for detecting human 5-HT3-B activity involve the direct measurement of human 5-HT3-B activity in whole cells transfected with human 5-HT3-A receptor with or without 5-HT3-B cDNA or oocytes injected with human 5-HT3-A receptor and 5-HT3-B mRNA. Human 5-HT3-B activity is measured by specific ligand binding and biological characteristics of the host cells

expressing human 5-HT3-B DNA. In the case of recombinant host cells expressing human 5-HT3-A receptor and human 5-HT3-B, patch voltage clamp techniques can be used to measure receptor activity and quantitate human 5-HT3-B protein. In the case of oocytes patch clamp as well as two-electrode voltage clamp techniques can be used to measure the decay rate of agonist-induced currents or agonist dose response.

Levels of human 5-HT3-B protein in host cells are quantitated by immunoaffinity. Cells expressing h5-HT5-B can be assayed for the number of cell surface receptor molecules expressed by measuring the amount of radioactive mCPBG binding to cell membranes. Human 5-HT3-B -specific affinity beads or human 5-HT3-B -specific antibodies are used to isolate for example ^{35}S-methionine labelled or unlabelled human 5-HT3-B protein. Labelled human 5-HT3-B protein is analyzed by SDS-PAGE. Unlabelled human 5-HT3-B protein is detected by Western blotting, ELISA or RIA assays employing human 5-HT3-B specific antibodies.

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human 5-HT3-B sequence but will be capable of hybridizing to human 5-HT3-B DNA even in the presence of DNA oligonucleotides with mismatches under appropriate conditions. Under alternate conditions, the mismatched DNA oligonucleotides may still hybridize to the human 5-HT3-B DNA to permit identification and isolation of human 5-HT3-B encoding DNA.

DNA encoding human 5-HT3-B from a particular organism may be used to isolate and purify homologues of human 5-HT3-B from other organisms. To

accomplish this, the first human 5-HT3-B DNA may be mixed with a sample containing DNA encoding homologues of human 5-HT3-B under appropriate hybridization conditions. The hybridized DNA complex may be isolated and the DNA encoding the homologous DNA may be purified therefrom.

It is known that there is a substantial amount of redundancy in the various codons that code for specific amino acids. Therefore, this invention is also directed to those DNA sequences that contain alternative codons that code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein that does not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of human 5-HT3-B is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of human 5-HT3-B. The term "functional derivatives" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" or to "chemical derivatives" of human 5-HT3-B. The term "fragment" is meant to refer to any polypeptide

subset of human 5-HT3-B. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire human 5-HT3-B molecule or to a fragment thereof. A molecule is "substantially similar" to human 5-HT3-B if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical. The term "analog" refers to a molecule substantially similar in function to either the entire human 5-HT3-B molecule or to a fragment thereof.

Following expression of human 5-HT3-B in a recombinant host cell, human 5-HT3-B protein may be recovered to provide human 5-HT3-B in active form. Several serotonin 5-HT3-A receptor purification procedures are available and suitable for use (Fletcher and Barnes, 1997; Fletcher et al., 1998; Lummis and Martin, 1992; Miller et al., 1992). As described above for purification of human 5-HT3-B from natural sources, recombinant human 5-HT3-B may be purified from cell lysates and extracts, or from conditioned culture medium, by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography.

In addition, recombinant human 5-HT3-B can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for full length nascent human 5-HT3-B, polypeptide fragments of human 5-HT3-B or human 5-HT3-B subunits.

Monospecific antibodies to human 5-HT3-B are purified from mammalian antisera containing antibodies reactive against human 5-HT3-B or are prepared as

monoclonal antibodies reactive with human 5-HT3-B using the technique of Kohler and Milstein, *Nature* 256: 495-497 (1975). Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for human 5-HT3-B. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the human 5-HT3-B, as described above. Human 5-HT3-B specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits being preferred, with an appropriate concentration of human 5-HT3-B either with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of peptide encoding a fragment of human 5-HT3-B associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing Corynebacterium parvum and tRNA. The initial immunization consists of human 5-HT3-B peptide in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of the antigen in Freund's incomplete adjuvant by the same route. Booster injections are given at about three-week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about -20°C.

Monoclonal antibodies (mAb) reactive with human 5-HT3-B are prepared by immunizing inbred mice, preferably Balb/c, with human 5-HT3-B peptide.

The mice are immunized by the IP or SC route with about 0.1 mg to about 10 mg, preferably about 1 mg, of human 5-HT3-B peptide in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed above. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 0.1 to about 10 mg of human 5-HT3-B polypeptide in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions that will allow the formation of stable hybridomas. Fusion partners may include, but are not limited to: mouse myelomas P3/NS1/Ag 4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being generally preferred. The antibody producing cells and myeloma cells are fused in polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days 14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using human 5-HT3-B peptide as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a techniques such as the soft agar technique MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

Monoclonal antibodies are produced *in vivo* by injection of pristane primed Balb/c mice, approximately 0.5 ml per mouse, with about 2×10^6 to about 6×10^6 hybridoma cells about 4 days after priming. Ascites fluid is collected at

approximately 8-12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of anti- human 5-HT3-B mAb is carried out by growing the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of human 5-HT3-B in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the above described methods for producing monospecific antibodies may be utilized to produce antibodies specific for human 5-HT3-B polypeptide fragments, or full-length nascent human 5-HT3-B polypeptide, or the individual human 5-HT3-B subunits. Specifically, it is readily apparent to those skilled in the art that monospecific antibodies may be generated which are specific for only the human 5-HT3-B subunit or the fully functional receptor.

Human 5-HT3-B antibody affinity columns can be made by adding the antibodies to Affigel-10 (Bio-Rad), a gel support which is activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activate esters are then quenched with ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous

protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing human 5-HT3-B subunits are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A_{280}) falls to background, then the protein is eluted with 0.23 M glycine-HC1 (pH 2.6). The purified human 5-HT3-B protein is then dialyzed against phosphate buffered saline.

DNA clones, termed p5HT3BR, are identified which encode proteins that, when expressed in any recombinant host, including but not limited to mammalian cells or insect cells or bacteria form a human 5-HT3-B sensitive to serotonin when co-expressed with 5-HT3-A receptor subunits. The expression of human 5-HT3-B DNA results in the reconstitution of the properties observed in oocytes injected with human 5-HT3-B -encoding poly $(A)^+$ RNA together with 5-HT3-A receptor subunits. These include: modification of the 5-HT-, mCPBG and 1-PBG-induced responses compared to those observed for 5-HT3-A homomultimers.

Serotonin is a biogenic amine transmitter that functions in some capacity in many physiological and pathophysiological conditions. Serotonin acts as a neurotransmitter and neuromodulator in the central and peripheral nervous systems, mediates inflammatory and allergic responses, regulates airway function, controls acid secretion in the stomach, regulates cardiovascular function as well as arterial and venous responses and is likely involved in to processes yet to be determined. The serotonin receptors that mediate these include the ligand-gated 5-HT3 receptor. Overlap of 5-HT3-A and 5-HT3-B receptor expression suggests that the putative heteromultimer is involved in central and peripheral nervous system as well as small intestine, thymus, prostate and uterine function. One way to understand which serotonin receptors are involved in these processes is to develop chemical modulators of the receptors as research tools and therapeutic entities. Recombinant host cells expressing the human serotonin 5-HT3-A and human 5-HT3-B receptors can be used to provide materials for a screening

method to identify such agonists and antagonists. As such, this invention of the human serotonin 5-HT3-B subunit directly teaches a way to identify new agonists and antagonists that may prove useful as research tools or may be used as therapeutics to treat disorders directly or indirectly involving serotonin receptors.

The present invention is also directed to methods for screening for compounds that modulate the expression of DNA or RNA encoding human 5-HT3-B as well as the function of human 5-HT3-B protein *in vivo*. Compounds that modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding human 5-HT3-B, or the function of human 5-HT3-B protein. Compounds that modulate the expression of DNA or RNA encoding human 5-HT3-B or the function of human 5-HT3-B protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Modulators identified in this process are useful as therapeutic agents, research tools and diagnostic agents.

Kits containing human 5-HT3-B DNA or RNA, antibodies to human 5-HT3-B, or human 5-HT3-B protein may be prepared. Such kits are used to detect DNA that hybridizes to human 5-HT3-B DNA or to detect the presence of human 5-HT3-B protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic analyses, diagnostic applications, and epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of human 5-HT3-B DNA, human 5-HT3-B RNA or human 5-HT3-B protein. The

recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of human 5-HT3-B. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant human 5-HT3-B protein or anti- human 5-HT3-B antibodies suitable for detecting human 5-HT3-B. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Nucleotide sequences that are complementary to the human 5-HT3-B encoding DNA sequence can be synthesized for antisense therapy. These antisense molecules may be DNA, stable derivatives of DNA such as phosphorothioates or methylphosphonates, RNA, stable derivatives of RNA such as 2'-*O*-alkylRNA, or other human 5-HT3-B antisense oligonucleotide mimetics. Human 5-HT3-B antisense molecules may be introduced into cells by microinjection, liposome encapsulation or by expression from vectors harboring the antisense sequence. Human 5-HT3-B antisense therapy may be particularly useful for the treatment of diseases where it is beneficial to reduce human 5-HT3-B activity.

Human 5-HT3-B gene therapy may be used to introduce human 5-HT3-B into the cells of target organisms. The human 5-HT3-B gene can be ligated into viral vectors that mediate transfer of the human 5-HT3-B DNA by infection of recipient host cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, poliovirus and the like. Alternatively, human 5-HT3-B DNA can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted DNA transfer using ligand-DNA conjugates or adenovirus-ligand-DNA conjugates, lipofection membrane fusion or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* human 5-HT3-B gene therapy.

Human 5-HT3-B gene therapy may be particularly useful for the treatment of diseases where it is beneficial to elevate human 5-HT3-B activity.

Pharmaceutically useful compositions comprising human 5-HT3-B DNA, human 5-HT3-B RNA, or human 5-HT3-B protein, or modulators of human 5-HT3-B receptor activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders in which modulation of human 5-HT3-B -related activity is indicated. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties that are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages defined by routine testing in order to obtain optimal

inhibition of the human 5-HT3-B receptor or its activity while minimizing any potential toxicity. In addition, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds or modulators identified according to this invention as the active ingredient for use in the modulation of human 5-HT3-B receptors can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds or modulators can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts. An effective but non-toxic amount of the compound desired can be employed as a serotonin 5-HT3-A/5-HT3-B receptor modulating agent.

The daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per patient, per day. For oral administration, the compositions are preferably provided in the form of scored or unscored tablets containing 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, and 50.0 milligrams of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. An effective amount of the drug is ordinarily supplied at a dosage level of from about 0.0001 mg/kg to about 100 mg/kg of body weight per day. The range is more particularly from about 0.001 mg/kg to 10 mg/kg of body weight per day. The dosages of the human 5-HT3-B receptor modulators are adjusted when combined to achieve desired effects. On the other hand, dosages of these various

agents may be independently optimized and combined to achieve a synergistic result wherein the pathology is reduced more than it would be if either agent were used alone.

Advantageously, compounds or modulators of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds or modulators for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds or modulators of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

In the methods of the present invention, the compounds or modulators herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include, without limitation, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. Disintegrators include, without limitation, starch, methylcellulose, agar, bentonite, xanthan gum and the like.

For liquid forms the active drug component can be combined in suitably flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methyl-cellulose and the like. Other dispersing agents that may be employed include glycerin and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations, which generally contain suitable preservatives, are employed when intravenous administration is desired.

Topical preparations containing the active drug component can be admixed with a variety of carrier materials well known in the art, such as, e.g., alcohols, aloe

vera gel, allantoin, glycerine, vitamin A and E oils, mineral oil, PPG2 myristyl propionate, and the like, to form, e.g., alcoholic solutions, topical cleansers, cleansing creams, skin gels, skin lotions, and shampoos in cream or gel formulations.

The compounds or modulators of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

Compounds of the present invention may also be delivered by the use of monoclonal antibodies as individual carriers to which the compound molecules are coupled. The compounds or modulators of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropylmethacryl-amidephenol, polyhydroxy-ethylaspartamidephenol, or polyethyl-eneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds or modulators of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydro-pyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

For oral administration, the compounds or modulators may be administered in capsule, tablet, or bolus form or alternatively they can be mixed in the animals feed. The capsules, tablets, and boluses are comprised of the active ingredient in combination with an appropriate carrier vehicle such as starch, talc, magnesium stearate, or di-calcium phosphate. These unit dosage forms are prepared by intimately mixing the active ingredient with suitable finely-powdered inert ingredients including diluents, fillers,

disintegrating agents, and/or binders such that a uniform mixture is obtained. An inert ingredient is one that will not react with the compounds or modulators and which is non-toxic to the animal being treated. Suitable inert ingredients include starch, lactose, talc, magnesium stearate, vegetable gums and oils, and the like. These formulations may contain a widely variable amount of the active and inactive ingredients depending on numerous factors such as the size and type of the animal species to be treated and the type and severity of the infection. The active ingredient may also be administered as an additive to the feed by simply mixing the compound with the feedstuff or by applying the compound to the surface of the feed. Alternatively the active ingredient may be mixed with an inert carrier and the resulting composition may then either be mixed with the feed or fed directly to the animal. Suitable inert carriers include corn meal, citrus meal, fermentation residues, soya grits, dried grains and the like. The active ingredients are intimately mixed with these inert carriers by grinding, stirring, milling, or tumbling such that the final composition contains from 0.001 to 5% by weight of the active ingredient.

The compounds or modulators may alternatively be administered parenterally via injection of a formulation consisting of the active ingredient dissolved in an inert liquid carrier. Injection may be either intramuscular, intraruminal, intratracheal, or subcutaneous. The injectable formulation consists of the active ingredient mixed with an appropriate inert liquid carrier. Acceptable liquid carriers include the vegetable oils such as peanut oil, cottonseed oil, sesame oil and the like as well as organic solvents such as solketal, glycerol formal and the like. As an alternative, aqueous parenteral formulations may also be used. The vegetable oils are the preferred liquid carriers. The formulations are prepared by dissolving or suspending the active ingredient in the liquid carrier such that the final formulation contains from 0.005 to 10% by weight of the active ingredient.

Topical application of the compounds or modulators is possible through the use of a liquid drench or a shampoo containing the instant compounds or modulators as an aqueous solution or suspension. These formulations generally contain a suspending agent such as bentonite and normally will also contain an antifoaming agent. Formulations containing from 0.005 to 10% by weight of the active ingredient are acceptable. Preferred formulations are those containing from 0.01 to 5% by weight of the instant compounds or modulators.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1:

Cloning of p5HT3BR

cDNA synthesis

First strand synthesis: Approximately 5 μg of human small intestine mRNA (Clontech) was used to synthesize cDNA using the cDNA synthesis kit (Life Technologies). 2 μl of NotI primer adapter was added to 5μl of mRNA and the mixture was heated to 70 ^{0} C for 10 minutes and placed on ice. The following reagents were added on ice: 4μl of 5x first strand buffer (250mM TRIS-HCl (pH8.3), 375mM KCl, 15mM$MgCl_2$), 2μl of 0.1M DTT, 10mM dNTP (nucleotide triphosphates) mix and 1μl of DEPC treated water. The reaction was incubated at 42 ^{0}C for 5minutes. Finally, 5μl of Superscript RT II was added and incubated at 42 °C for 2 more hours. The reaction was terminated on ice.

Second strand synthesis: The first strand product was adjusted to 93μl with water and the following reagents were added on ice: 30 μl of 5x 2nd strand buffer (100 mM TRIS-HCl (pH6.9), 450 mM KCl, 23 mM $MgCl_2$, 0.75 mM β-NAD+, 50mM $(NH_4)_2SO_4$), 3μl of 10 mM dNTP (nucleotide triphosphates), 1μl E. coli DNA

ligase (10units) 1µl RNase H (2units), 4 µl DNA pol I (10 units). The reaction was incubated at 16°C for 2 hours. The DNA from second strand synthesis was treated with T4 DNA polymerase and placed at 16°C to blunt the DNA ends. The double stranded cDNA was extracted with 150 µl of a mixture of phenol and chloroform (1:1, v:v) and precipitated with 0.5 volumes of 7.5 M NH4OAc and 2 volumes of absolute ethanol. The pellet was washed with 70% ethanol and dried down at 37°C to remove the residual ethanol. The double stranded DNA pellet was resuspended in 25 µl of water and the following reagents were added; 10 µl of 5x T4 DNA ligase buffer, 10 µl of Sall adapters and 5 µl of T4 DNA ligase. The ingredients were mixed gently and ligated overnight at 16° C. The ligation mix was extracted with phenol:chloroform:isoamyl alcohol, vortexed thoroughly and centrifuged at room temperature for 5 minutes at 14,000 x g to separate the phases. The aqueous phase was transferred to a new tube and the volume adjusted to 100 ml with water. The purified DNA was size selected on a chromaspin 1000 column (Clontech) to eliminate the smaller cDNA molecules. The double stranded DNA was digested with NotI restriction enzyme for 3-4 hours at 37° C. The restriction digest was electrophoresed on a 0.8 % low melt agarose gel. The cDNA in the range of 1-5 KB was cut out and purified using Gelzyme (InVitrogen). The product was extracted with phenol:chloroform and precipitated with NH_4OAc and absolute ethanol. The pellet was washed with 70% ethanol and resuspended in 10 ml of water.

Ligation of cDNA to the Vector: The cDNA was split up into 5 tubes (2µl each) and the ligation reactions were set up by adding 4.5 µl of water, 2 µl of 5x ligation buffer, 1µl of p-Sport vector DNA (cut with Sal-1 / NotI and phosphatase treated) and 0.5 µl of T4 DNA ligase. The ligation was incubated at 40° C overnight.

Introduction of Ligated cDNA into E. coli by Electroporation:

The ligation reaction volume was adjusted to a total volume of 20 µl with water. Five ml of yeast tRNA, 12.5 ml of 7.5M NH_4OAc and 70 ml of absolute ethanol (-20°C) was added. The mixture was vortexed thoroughly, and immediately centrifuged at room temperature for 20 minutes at 14,000 x g. The pellets were washed in 70% ethanol and each pellet was resuspended in 5 ml of water. All 5 ligations (25ml) were pooled and 100µl of DH10B electro-competent cells (Life Technologies) were electroporated with 1 ml of DNA (total of 20 electroporations), then plated out on ampicillin plates to determine the number of recombinants (cfu) per µl. The entire library was seeded into 2 liters of Super-Broth and maxipreps were made using Promega Maxi Prep kit and purified on cesium chloride gradients.

Screening of library:

One-microliter aliquots of the library constructed above were electroporated into Electromax DH10B cells (Life Technologies). The volume was adjusted to 1 ml with SOC media and incubated for 1 hour at 37°C with shaking. The library was then plated out on 50 150cm^2 plates containing LB to a density of 5000 colonies per plate. These were grown overnight at 37°C.

A probe to 5-HT3-B was generated by polymerase chain reaction using the following primer pair:

5' oligo: 5' GAT CTC CCT ACC TCT AAG TG 3' {SEQ.ID. NO.: 1]
3' oligo: 5' AGC ACA CTG GTC TTG AAC AC 3' [SEQ.ID.NO. 2].

Amplification was cycled 35 times using 50-60°C annealing temperature and a human small intestine cDNA as template. The PCR fragment that was generated (400-500 bp) was 32P-labelled using the Klenow fragment of DNA polymerase I and an oligo labeling kit (Pharmacia). The fragment was then cleaned by one passage through a S-200 column (Pharmacia).

The library colonies are lifted on nitrocellulose filters and cross-linked via UV irradiation (Stratagene). Filters were washed three times in buffer (50 mM TRIS, 1 M NaCl, 2mM EDTA, 1% SDS) at 42°C. Filters were then prehybridized in 1:1 Southern Prehyb:Formamide with salmon sperm DNA (50mg, boiled) for 6 hours at 42°C. Filters were then hybridized with the probe ($1x10^6$ counts/ml) overnight. The filters were then washed one time with 2xSSC/0.2%SDS at room temperature for 15 minutes, 2 times with 0.2xSSC/0.1%SDS at 45°C for 30 minutes each. Filters were then wrapped in plastic wrap and exposed to film (Kodak) overnight at -80°C.

Positive clones were identified. Resulting positives were cored from the original plate, incubated in LB for 45 minutes at 37°C and re-plated overnight. The filter lifting/hybridizing/washing/colony picking procedure was replicated until a single clone or clones were isolated, representing an individual cDNA.

From the screen for human novel 5HT3-like receptor, all cDNA clones were isolated and sequenced. One clone, pH3R, contained a 2699 bp insert (Figure 1). This sequence had an apparent open reading frame from nucleotide 299 to 1335 (Figure 2). This open reading frame encoded a protein of 445 amino acids (Figure 3).

EXAMPLE 2

Cloning of 5-HT3-B cDNA into a Mammalian Expression Vector

The 5-HT3-B cDNAs (collectively referred to as p5HT3BR) were cloned into the mammalian expression vector pcDNA3.1zeo(+) (InVitrogen). The 5-HT3-B cDNA clone was isolated from a human small intestine cDNA library. The full-length cDNA was used as the template for PCR using specific primers with BamHI (5'AAC GTT GAA TTC GCC ACC ATG TTG TCA AGT GTA ATG GCT CCC CTG TGG GCC3') [SEQ.ID.NO. 3] and HindIII (5'AAC GTT AAG CTT TCT TAA GTG CCA GCA CAA TTA CTT GAA G 3') [SEQ.ID.NO. 4] sites for

cloning. The PCR product was purified on a column (Wizard PCR DNA purification kit from Promega) and digested with NheI and NotI (NEB) to create cohesive ends. The product was purified by a low melting agarose gel electrophoresis. The pcDNA3.1zeo(+) vector was digested with NheI and NotI enzymes and subsequently purified on a low melt agarose gel. The linear vector was used to ligate to the 5-HT3-B cDNA inserts. Recombinants were isolated, designated 5-HT3-B, and used to transfect mammalian cells stably expressing the human 5-HT3-A receptor transfected in a pCIneo vector (using EcoRI and XbaI cloning sites) (5-HT3-A/HEK293 cells) by electroporation. Stable cell clones were selected by growth in the presence of G418 and zeocin. Single G418/zeocin resistant clones were isolated and shown to contain the intact 5-HT3-B gene. Clones containing the human 5-HT3-B cDNAs were analyzed for p5HT3BR expression by measuring Ca^{2+} influx using Fluo-4 in response to serotonin and 1-PBG (Figure 9 a, b, c). Responses were compared to those obtained from 5-HT3-A-expressing HEK293 cells.

Cells stably expressing human 5-HT3-B together with human 5-HT3-A were used to test for expression human 5-HT3-B and for functional activity. These cells are used to identify and examine other compounds for their ability to modulate, inhibit or activate the human 5-HT3-B. Other cells expressing both 5-HT3-A and 5-HT3-B subunits can be used to identify and examine other compounds for their ability to modulate, inhibit or activate the human 5-HT3-B.

Cassettes containing the human 5-HT3-B cDNA in the positive orientation with respect to the promoter are ligated into appropriate restriction sites 3' of the promoter and identified by restriction site mapping and/or sequencing. These cDNA expression vectors are introduced into fibroblastic host cells for example HEK293 by standard methods including but not limited to electroporation, or chemical procedures (cationic liposomes, DEAE dextran, calcium phosphate).

All of the vectors used for mammalian transient expression can be used to establish stable cell lines expressing the human 5-HT3-B. Unaltered human 5-HT3-B constructs cloned into expression vectors are expected to program host cells to make human 5-HT3-B protein. The transfection host cells include, but are not limited to, HEK293, CV-1-P [Sackevitz et al., Science 238: 1575 (1987)], tk-L [Wigler, et al. Cell 11: 223 (1977)], NS/0, and dHFr- CHO [Kaufman and Sharp, J. Mol. Biol. 159: 601, (1982)].

Co-transfection of any vector containing the human 5-HT3-B cDNA with a drug selection plasmid including, but not limited to G418, zeocin, aminoglycoside phosphotransferase; hygromycin, hygromycin-B phosphotransferase; APRT, xanthine-guanine phosphoribosyl-transferase, will allow for the selection of stably transfected clones. Levels of the human 5-HT3-B are quantitated by the assays described herein.

The human 5-HT3-B cDNA constructs are also ligated into vectors containing amplifiable drug-resistance markers for the production of mammalian cell clones synthesizing the highest possible levels of the human 5-HT3-B. Following introduction of these constructs into cells, clones containing the plasmid are selected with the appropriate agent, and isolation of an over-expressing clone with a high copy number of plasmids is accomplished by selection in increasing doses of the agent.

The expression of recombinant human 5-HT3-B is achieved by transfection of full-length the human 5-HT3-B cDNA into a mammalian host cell.

EXAMPLE 3

Primary Structure of the Human 5-HT3-B Protein

The nucleotide sequences of p5HT3BR revealed single large open reading frame of about 1326 base pairs as shown in Figure 2. The cDNAs have 5' and 3'-

untranslated extensions of about 141 and about 456 nucleotides for p5-HT3-BR. The first in-frame methionine was designated as the initiation codon for an open reading frame that predicts a human 5-HT3-B protein with an estimated molecular mass (M_r) of about 50.3 kDa. The protein contained hydrophobic amino terminal residues with sequences highly predictive of signal cleavage sites that would result in mature proteins initiating at amino acid 22.

The predicted human 5-HT3-B protein was aligned with nucleotide and protein databases and found to be related to the known 5-HT3-A receptors. Approximately 70% of the amino acids in 5-HT3-B were highly conserved, showing at least 44% amino acid identity within the serotonin 5-HT3 family of receptor. The conserved motifs found in this family of receptor, such as the 4 putative transmembrane domains with similar spacing, were also found in the human 5-HT3-B sequence. The identity of the 5-HT3-B receptor with the 5-HT3-A receptor at the nucleotide level was only about 60%. The human 5-HT3-B protein contained the conserved cysteine residues found in the conserved cysteine-cysteine loop that may form the agonist-binding site of ligand-gated ion channels (Lambert et al., 1995). There is strong homology to the proposed ligand recognition site in the first N-terminal loop in the murine 5-HT3-A and the nicotinic AChR α7 [xIWxPDILxxExxD]; the only difference in the shown "consensus" in the 5-HT3-B protein is a conserved change: L119 to I119. The E106 in the 5-HT3-A (murine) is critical for high affinity 5-HT binding (Boess et al., 1997).

Five potential sites of glycosylation (Marshall, 1972) were located at the extracellular amino terminus and 1 potential site for protein kinase C (Woodgett et la., 1986), 3 potential sites for casein kinase II (Pinna, 1990), and 1 site for mammary gland casein kinase were located in the cytoplasmic loop between M3 and M4 as shown in Figure 3.

EXAMPLE 4

Distribution of 5-HT3-B mRNA.

The tissue distribution of 5-HT3-B mRNAs was determined by semi-quantitative PCR. A primer set specific to 5-HT3-B (TGTGTTCAAGACCAGTGTGC [SEQ.ID.NO.8]; TAGCTTTGGAAGAGCAGTCG [SEQ.ID.NO.9]) was used to complete amplification of a portion of the 5-HT3-B mRNA via PCR using cDNAs templates synthesized from poly (A) RNA (Clontech, Palo Alto, CA) which was extracted from various human tissues (tissue types shown in Figure 4a). To gain increased specificity and sensitivity, an oligonucleotide (TGTTGGTCAAATTCCTCCATGATGAGCAGCGTGGTGGACA [SEQ.ID.NO.10]) was phosphorylated using γ-^{32}P-ATP with polynucleotide kinase as described by manufacturer (Amersham Pharmacia Biotech, Piscataway, New Jersey) and annealed to denatured PCR products and resolved by 6% polyacrylamide gel electrophoresis. The subsequent gel was then dried down and imaged (PhosphorImager 445SI, Molecular Dynamics).

As shown in figure 4a, PCR-based tissue distribution analysis reveals that the 5-HT3-B mRNA is expressed in human cerebral cortex including occipital, frontal and temporal regions, amygdala, hippocampus, testis. Very low levels were observed in adrenal gland, bone marrow, lymph node, salivary gland, thyroid. No detectable transcript was observed in heart, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, ovary, small intestine, colon, leukocytes, cerebellum, medulla, spinal cord, putamen, caudate nucleus, corpus callosum, substantia nigra, and thalamus.

In the CNS, the 5-HT3 receptor is found in high density in nuclei of the lower brainstem, area postrema and nucleus of the tractus solitarius. Lower densities of the receptor are found in the cerebral cortex and limbic areas, including the hippocampus. In the periphery, 5-HT3 receptors are located on pre-

and postganglionic neurons of both sensory and enteric nervous systems (Eglen and Bonhaus, 1996). Northern analysis revealed some overlap of 5-HT3-B and 5-HT3-A receptor distributions.

Figure 4b shows RT-PCR in situ hybridization that was performed on normal human tissues of the spleen (b1, b2) and small intestine (b3, b4). Human spleen had intense 5-HT3-B mRNA (b1) and 5HT3-A mRNA (b2) signal (purple in color – dark grey in black-and-white), respectively, in small lymphocytes (arrows). Similarly, intense 5-HT3-B mRNA (b3) and 5HT3-A mRNA (b4) signal (purple) was detected in stromal fibroblasts of the small intestines (arrows). Total magnification = 600x.

Figure 4c shows RT-PCR in situ hybridization that was performed on serial 10 μm sections of the normal monkey amygdala. Figures 4c1 (300x) and 4c3 (600x) show positive 5-HT3-A mRNA signal (purple in color – dark grey in black-and-white) in seven neurons (arrows) which is also show positive 5HT3-B mRNA signal (4c2 (300x); 4c4 (600x)). Not all cells were labeled by the RT-PCR ISH technique using specific probes for 5-HT3-B or 5-HT3-A.

EXAMPLE 5

Characterization of the Function of protein encoded by p5HT3BR in Xenopus oocytes

Xenopus laevis oocytes were prepared and injected using standard methods previously described and known in the art (Fraser et al., 1993). Ovarian lobes from adult female *Xenopus laevis* (Nasco, Fort Atkinson, WI) were teased apart, rinsed several times in nominally Ca-free saline containing (in mM): NaCl 82.5, KCl 2.5, $MgCl_2$ 1, HEPES 5, adjusted to pH 7.0 with NaOH (OR-2), and gently shaken in OR-2 containing 0.2% collagenase Type 1 (ICN Biomedicals, Aurora, Ohio) for 2-5 hours. When approximately 50% of the follicular layers were removed, Stage V and VI oocytes were selected and rinsed in media consisting of 75% OR-2 and 25% ND-

96. The ND-96 contained (in mM): NaCl 100, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, Na pyruvate 2.5, gentamicin (50 ug/ml), adjusted to pH 7.0 with NaOH. The extracellular Ca^{2+} was gradually increased and the cells were maintained in ND-96 for 2-24 hours before injection. For in vitro transcription, pGEM HE (Liman et al., 1992)) containing human 5-HT3-A (Genbank D49394) or 5-HT3-B cDNA was linearized with NheI and transcribed with T7 or SP6 RNA polymerase (Stratagene) in the presence of the cap analog m7G(5')ppp(5')G. The synthesized cRNA was purified with a Sephadex G-50 spin column. Oocytes were injected with 50 nl of the human serotonin 5-HT3-A receptor with or without the 5-HT3-B RNA (0.02 and 0.002-0.2 ng each) or other channel or receptor subunit. Control oocytes were injected with 50 nl of water. Oocytes were incubated for 2-10 days in ND-96 before analysis for expression of the human 5-HT3-B. Incubations and collagenase digestion were carried out at room temperature. Injected oocytes were maintained in 48 well cell culture clusters (Costar, Cambridge, MA) at 18°C. Whole cell agonist-induced currents were measured 1-14 days after injection with a conventional two-electrode voltage clamp (GeneClamp500, Axon Instruments, Foster City, CA) using standard methods previously described and known in the art (Dascal, 1987). The microelectrodes were filled with 3 M KCl, which had resistances of 1 and 2 MΩ. Cells were continuously perfused with ND96 at 10 ml/min at room temperature unless indicated. Membrane voltage was clamped at -88 mV unless indicated.

5-HT (≥100 μM) had no effect in oocytes injected with putative 5-HT3-B subunit alone (n= 4 (3.3 ng cRNA/oocyte), n= 12 (0.33 ng), n= 12 (0.033 ng), and n=3 (0.0033 ng)) indicating there were no endogenous 5-HT-induced currents in the oocytes used in these studies. Oocytes injected with only 5-HT3-B cRNA (3.3 ng) or 5-HT3-B together with nACh β1, β2 and β3 were insensitive to 300 neuroactive compounds at ≥ 100 μM including 5-HT, ACh, histamine, tyramine, tryptamine, tryptophanamide, tryptophan, norepinephrine, octopamine, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid, tryptophol, alpha-methyl serotonin, glutamate, glycine, GABA, β-alanine, taurine, β-phenylethylamine, 5-

hydroxyindolacetic acid, 5-hydroxyindole, 6-hydroxymelatonin, gamma hydroxybutyrate, cis-4 aminocrotonic acid, agmatine, d-cycloserine, N-acetyl-L-cysteine, acetyl-aspartyl-L-glutamic acid, S-α-histamine, N-α-methyl histamine, melatonin, 5-hydroxyindole 2-carboxylic acid, N-acetyl serotonin, and 5-hydroxyindole 3-acetamide. Injection of 5-HT3-B-injected oocytes with bacterial alkaline phosphatase (0.25- 0.3 U) at least 30 min prior to recording was ineffective in conferring sensitivity to these ligands (n=3).

Three characteristics of the 5-HT3-A receptor agonist-induced responses were dramatically altered in 5-HT3-B-injected oocytes. First, the kinetics of the 5-HT-induced response were markedly altered (Figure 5a); second, peak currents were increased (Figure 5c); and third, 5-HT3-B specifically modified the dose-response relationship to 5-HT, the biphenylguanide derivatives mCPBG and 1-PBG, and 2-Me-5-HT, with no detectable effect on the dose-response to dopamine (DA).

Functional expression of human serotonin 5-HT3 receptor modifier subunit 5-HT3-B together with 5-HT3-A receptor in Xenopus oocytes is shown in Figure 5. Oocytes were continuously perfused with Ba^{2+} containing ND96 at a rate of 10 ml/min at room temperature. The inward current through 5-HT3-A homomeric receptors declined during the continued presence of 1 to 100 μM 5-HT as shown in Figure 5a, consistent with the desensitization previously described for this receptor (Belelli et al., 1995; Hope et al., 1996; Lankiewicz et al., 1998). 5-HT3-A-expressing oocytes responded to 5-HT, 2-methyl-5-HT, mCPBG and 1-PBG with either a slowly desensitizing response (left panel; "slow" responders, 71% of oocyte batches), or a complex current that included a rapidly desensitizing component (right panel; "fast" responders). Oocytes expressing both subunits produced uniform responses (middle panel). The agonists tested included 5-HT (0.3 and 10 μM, responses are superimposed); mCPBG (0.3 and 10 μM); 1-PBG (10 and 100 μM); DA (0.1 and 1 mM); 2-Me-5-HT (10 μM). Agonists are applied during the time indicated by the horizontal

bar above the record. The clear bar extending from the solid bar indicates the longer exposure to agonist for one of the superimposed traces. Time scale bar: 40 sec.

Oocytes that had been co-injected with equivalent amounts of 5-HT3-A and 5-HT3-B cRNA always responded similarly, despite radical differences in response kinetics of the two populations of control 5-HT3-A-injected oocytes (Table 1). Thus, in "slow" oocytes, near-maximal 5-HT-induced currents decayed 5 times more quickly (Figure 5, left vs. middle panels; Figure 6a; Table 1), while in "fast" oocytes, responses decayed 3.5-fold more slowly (Figure 5, right vs. middle panels; Figure 6b; Table 1). Furthermore, the time to peak for responses to 5-HT (10 μM) was 3.3 +/- 0.3 sec (n= 38) in the presence of 5-HT3-B for all oocytes tested (Figure 6c,d, solid bars, "slow" and "fast" responders). Thus, in the presence of 5-HT3-B, 5-HT3 receptor responses in oocytes were normalized to a moderately transient current. In contrast to the complex response waveforms observed in the absence of 5-HT3-B, inward currents elicited by 10 μM 5-HT in Ba^{2+} ND96 were best fit to a single exponential (τ=4.9 +/- 0.3 sec; n=25) in more than 80% of the oocytes expressing both subunits. This decay constant was significantly slower than the decay of "fast" responses under identical conditions ($p < 5e^{-6}$). In "slow" responders expressing 5-HT3-B, 5-HT, 2-Methyl-5-HT, mCPBG and 1-PBG decreased the t80 values by 5.6-fold, 5-fold, 2.0-fold and 1.7 fold, respectively, suggesting agonist-dependent differences (Table 1). In contrast, the 5-HT3-B modulatory effect on t80 values of "fast" responders to these agonists was about four-fold. Significant differences in t80 values were observed at 1 and 10 μM 5-HT in all oocyte batches. Interestingly, DA responses did not appear to be altered by 5-HT3-B co-expression (Figure 5a).

The response of homomeric and heteromeric receptors to 5-HT was consistent with the activation of non-selective cation channels since the current reversed near 0 mV. The differences in kinetics appeared to be due to alterations in 5-HT3 receptor function and not due to activation of contaminating endogenous Ca^{2+}-activated

chloride channels. While 5-HT3-A receptors are permeable to Ca^{2+} (Davies et al., 1999; Hargreaves et al., 1994; Ronde and Nichols, 1998; Yang, 1990), the influx of Ca^{2+} appears to be insufficient to activate endogenous chloride currents (Gilon and Yakel, 1995; Mair et al., 1998). Furthermore, 5-HT3-B diminishes the Ca^{2+} permeability of the human recombinant receptor (Davies et al., 1999). 5-HT3-B modulation of channel properties were observed in salines deplete of Ca^{2+}, and at membrane potentials near the chloride equilibrium potential.

Responses to 1-PBG were blocked by the 5-HT3 antagonist tropisetron. After obtaining a control response to 1-PGB (100 μM; indicated by the solid bar) (Figure 5b, left panel), the oocyte was washed 2 min then incubated for 30 sec in tropisetron (1 μM; clear bar) then challenged with both 1-PBG and tropisetron. The response to 1-PBG was completely blocked (Figure 5b, middle panel). The response to agonist recovered after a 2 min washout of antagonist (Figure 5b, right panel).

Co-expression of 5-HT3-A together with 5-HT3-B cRNA (solid bars, Figure 5c) significantly increased the maximum response to mCPBG, 1-PBG and 5-HT compared to expression with 5-HT3-A alone (grey bars, Figure 5c). Data from oocytes recorded in Ba^{2+}- and Ca^{2+}- containing saline were combined since there were no differences in 5-HT (10 μM)-induced maximal current in Ca^{2+}-containing vs. Ba^{2+}-containing saline (-7.7 +/- 1.4 μA (n=16) vs. -7.6 +/- 0.9 μA (n=24)). The number of oocytes tested ranged from 9 to 61; SEM values were 10% for 5-HT (10 μM) and 21% for both mCPBG (10 μM) and 1-PBG (100 μM). p values are indicated: * $p<0.05$, ** $p<0.01$ (Students t-test).

The desensitization of the response was quantified by measuring the time between 80% to peak on the rising and falling phases of the response (t80). The rate of decay was slower in salines in which Ca^{2+} was replaced by Ba^{2+}: in Ca^{2+}- and Ba^{2+}-containing saline, t80 was 11.3 +/- 2.6 sec (n= 18) and 30.3 +/- 4.3 sec (n= 31; p<

0.0005), respectively. Under no condition tested was t80 dependent on voltage. The initial decay of the "fast" responders could be fit by a single exponential with τ= 2.0 +/- 0.3 sec (n=8). Our quantification of kinetic parameters determined from oocyte currents provides a means of comparing response durations and onset between populations of oocytes treated under similar experimental conditions. It is clearly not an accurate description of the underlying channel activity because the relatively slow method of application and oocyte geometry precludes rapid exchange of solutions.

The time to peak of the response to 5-HT (10 μM) was 6.2 +/- 0.6 sec (n=41; Figure 6c) and 1.8 +/- 0.2 sec (n=10; Figure 6d) for slow and fast responders, respectively. Similar differences were obtained in response to 100 μM 5-HT. The t80 of homomeric "fast" responses was voltage independent in both in Ca^{2+}- and Ba^{2+}-containing salines similar to the finding with "slow" responses, however, in contrast to the latter, the t80 was similar in the presence and absence of Ca^{2+}.

The pharmacology of 5-HT3 receptors was modified by 5-HT3-B expression similarly in all batches of oocytes tested and the data were combined. The apparent affinity for 5-HT was decreased when 5-HT3-B was co-expressed with 5-HT3-A in oocytes (Figure 7a). On the other hand, oocytes expressing both subunits were more sensitive to application of low concentrations of mCPBG and 1-PBG compared to oocytes expressing 5-HT3-A alone (Figure 5a). Responses to low concentrations of DA were not enhanced by 5-HT3-B. When ratios of peak response to low and high concentrations of agonist were determined, co-injected oocytes had a larger relative response to the biphenylguanide derivatives (1-PBG and mCPBG) but smaller relative response to 5-HT and the magnitude of these differences depended on the relative ratio of cRNAs injected (Figure 7b). Consistent with the t80 value dependence on ratios of injected cRNA, the differences in pharmacology were no longer observed when 5-HT3-B cRNA was 100-fold more dilute than 5-HT3-A cRNA.

The agonists 1-PBG and mCPBG elicited no response in oocytes injected with 0.33 ng 5-HT3-B subunit alone (the concentration injected to give a 1:1 ratio; n=4),

indicating that the increase in 1-PBG and mCPBG responsiveness in co-injected oocytes was not due to a direct activation of 5-HT3-B homomultimers by these agonists. Furthermore, the response to 1-PBG was similarly blocked by tropisetron (1 μM), LY-278,584 maleate (1 μM), d-tubocurarine (30 μM) in a reversible manner. The selective 5-HT_2 receptor antagonist ketanserin (10 μM) had no effect on agonist responses. The 5-HT-induced response in co-injected oocytes was also blocked by these antagonists.

However, 5-HT3-B altered the voltage-dependence of agonist-induced currents such that they were linear rather than inward rectifying. In experiments in which the currents though 5-HT3 receptors were measured at a range of membrane voltages, 5-HT3-A receptors passed more inward current than outward current at voltages negative and positive to the reversal potential, respectively. In the presence of 5-HT3-B, inward and outward currents were similar and the current-voltage relationship was nearly linear. Injection of the 5-HT3-B RNA had no effect on currents through nACh receptors expressed in oocytes (Figure 8) as well as a *Shaker* potassium channel mutant lacking the N-terminal domain responsible for fast inactivation.

The specificity of the modulatory effect of human 5-HT3-B in oocytes is shown in Figure 8. In all cases the amount of injected 5-HT3-B cRNA was at least 2-fold the concentration of each nACh receptor. (a.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α3β4 with (solid; n= 5) and without (grey; n=4) 5-HT3-B. (b.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α4β2 with (solid; n=8) and without (grey; n=9) 5-HT3-B. (c.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α2β2 with (solid; n=3) and without (grey; n=3) 5-HT3-B. (d.) t80 for the response to 10 μM epibatidine exposure to oocytes expressing nACh receptor α7 with (solid; n=12) and without (grey; n=15) 5-HT3-B.

Furthermore, the responses to low and high concentrations of epibatidine (0.3 and 10 μM) were similar in α7 nACh-injected oocytes in the presence or absence of 5-HT3-B indicating that the dose response relationship was not appreciably altered.

EXAMPLE 6

Characterization of the Human 5-HT3-B

Human HEK293 cells stably expressing the human 5-HT3-A receptor were transfected with p5HT3BR. One double transfectant was similar to control 5-HT3-A/HEK cells and was usually used as a control. After three days the cells were selected in the presence of neomycin (500 μg/ml) and zeocin (200 μg/ml) and grown through three 1:10 splits for approximately two weeks. Individual colonies were picked and grown in 6-well dishes. Cells were then plated onto 96-well plates (Biocoat, poly-D-lysine coated black/clear plate, Becton Dickinson part # 354640) and grown to confluence for three days. Wells were rinsed with F12/DMEM, then incubated in Fluo-4 (2 μM) with Pluronic acid (20%, 40μl used in 20 ml total volume) for 1 hour at room temperature. Plates were assayed using the FLIPR (Molecular Devices, FL-101). Cells were challenged with agonists (at 3-fold concentration in 40 μl added to 80 μl at a velocity of 50 μl/sec).

The whole cell patch clamp technique (Hamill et al., 1981) was used to record ligand-induced currents from HEK293 stably expressing 5-HT3-A receptor or both the 5-HT3-A receptor and the 5-HT3-B protein maintained for >2 days on 12 mm coverslips. Cells were visualized using a Nikon Diaphot 300 with DIC Nomarski optics. Cells were continuously perfused in a physiological saline (~0.5 ml/min) unless otherwise indicated. The standard physiological saline ("Tyrodes") contained (in mM): 130 NaCl, 4 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, and 10 hemi-Na-HEPES (pH 7.3, 295-300 mOsm as measured using a Wescor 5500 vapor-pressure (Wescor, Inc., Logan, UT). Recording electrodes were fabricated from borosilicate capillary tubing (R6; Garner Glass, Claremont, CA), the tips were coated with dental periphery wax (Miles

Laboratories, South Bend, IN), and had resistances of 1-2 MΩ when containing intracellular saline (in mM: 100 K-gluconate, 25 KCl, 0.483 $CaCl_2$, 3 $MgCl_2$, 10 hemi-Na-HEPES and 1 K_4-BAPTA (100nM free Ca^{2+}); pH 7.4, with dextrose added to achieve 290 mOsm). Liquid junction potentials were -18 mV using standard pipette and bath solutions as determined both empirically and using the computer program JPCalc (Barry, 1994). All voltages shown are corrected for liquid junction potential. Current and voltage signals were detected and filtered at 2 kHz with an Axopatch 1D patch-clamp amplifier (Axon Instruments, Foster City, CA), digitally recorded with a DigiData 1200B laboratory interface (Axon Instruments), and PC compatible computer system and stored on magnetic disk for off-line analysis. Data acquisition and analysis were performed with PClamp software. Slow changes in holding current were detected and filtered at 2 kHz, and recorded with a LPF202A DC amplifier (Warner, Hamden, CT) and VR-10B digital data recorder (Instrutech, Great Neck, NY) onto video tape. The signal was later analyzed at 10 Hz using Axotape software.

The total membrane capacitance (C_m) was determined as the difference between the maximum current after a 30 mV hyperpolarizing voltage ramp from -68 mV generated at a rate of 10 mV/ms and the steady state current at the final potential (-98 mV) (Dubin et al., 1999).

Apparent reversal potentials (V_{rev}) of ligand-induced conductance changes were determined using a voltage-ramp protocol (Dubin et al., 1999). Voltage ramps were applied every 1 second and the resulting whole cell ramp-induced currents were recorded. Usually the voltage was ramped from negative to positive to negative values. The current required to clamp the cells at -68 mV was continuously monitored. Ligand-induced conductances were determined from whole-cell currents elicited by a voltage-ramp protocol in the presence and absence of ligand. Comparison of control ramp currents and those obtained in the presence of ligand reveals the difference between these currents and indicates the effect of the ligand on the channel protein. The voltage at which there was no net ligand-induced current was determined (V_{rev}).

Most values are presented as the arithmetic mean +/- standard error of the mean (S.E.M.).

Agonist-induced Ca^{2+} and ionic current responses had markedly faster decay kinetics in HEK293 cells stably transfected with both 5-HT3-A and 5-HT3-B subunits compared to cells stably expressing 5-HT3-A receptors (Figure 9). Cells expressing both 5-HT3-B and 5-HT3-A (Figure 9a, right) responded to 3 μM 5-HT (top, largest response), 30 μM 1-PBG (middle, largest response) and 3 μM mCPBG (bottom, largest response) with a faster decay compared to 5-HT3-A receptor-expressing cells (Figure 9a, left). These data are similar to those obtained from "slow" oocytes (Figure 5). Superimposed on the Ca^{2+} influx induced by high concentrations of agonist are responses of cells from the same plate to low concentrations of agonist (Figure 9a). As in the oocytes (Figure 7b), responses to low doses of mCPBG and 1-PBG were a larger percentage of the maximum response observed in doubly transfected cells (Figure 9c; $p< 0.005$ at 0.1 and 0.17 μM mCPBG). However, in contrast to the oocyte data (Figure 7a,b), low concentrations of 5-HT also elicited larger responses in double transfectants compared to 5-HT3-A/HEK (Figure 9b; $p < 0.005$ at 60, 100 and 170 nM 5-HT). Complete dose-response relationships for 5-HT and mCPBG (Figure 9b,c) as well as 1-PBG, 2-methyl 5-HT, 5HTQ, quipazine, DA and mCPP indicate a decreased nH and a higher affinity for agonists in 5-HT3-B-expressing cells (5-HT: EC50: 200 +/- 29 nM (n=8) compared to 540 +/- 50 nM (n=7), $p< 0.005$).

Dose response for 5-HT -activated Ca^{2+} influx using the FLIPR system is shown in Figure 9b. 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) had significantly higher affinity toward 5-HT and decreased nH compared to 5-HT3-A homomeric receptors (squares). Peak responses were determined and normalized to the maximum observed response. Mean values from 4-10 separate experiments are presented. Data were fit with Boltzmann exponentials.

Dose response for mCPBG-activated Ca^{2+} influx using the FLIPR system. Data from 5-HT3-A and 5-HT3-B heteromeric receptors (triangles) and 5-HT3-A homomeric receptors (squares) is shown in figure 9c. Peak responses were determined and normalized to the maximum observed response. Values were averaged from 4 separate experiments. Data were fit with Boltzmann exponentials.

Data acquired during fast application of agonist in the FLIPR system measuring receptor-mediated Ca^{2+} influx indicate that all three agonists behaved similarly: cells co-expressing both subunits revealed a shallower dose-response relationship shifted to higher affinity. A likely reason for the difference between the 5-HT dose response in oocytes and in recombinant mammalian cells is that the fast desensitization of the 5-HT response produced an apparent shift in affinity during the relatively slow application of 5-HT in the oocyte studies. This was not observed for 1-PBG and mCPBG, presumably because the responses to these agonists desensitized more slowly (Table 1).

TABLE 1

The t80 of current responses to prolonged exposure to 5-HT3 receptors is altered in *Xenopus* oocytes expressing heteromers of both 5-HT3-A and 5-HT3-B subunits. Mean +/- SEM (n) for peak responses obtained from a holding potential of -70 mV are shown. p values were determined using the Student's *t*-test.

	Oocytes expressing:	5-HT (10 μM)	2-Me-5-HT (10 μM)	mCPBG (10 μM)	1-PBG (100 μM)
Slow control 5-HT response	5-HT3-A	27.1 +/- 3/0 (38)	35 (1)	31.9 +/- 6.3 (9)	31.7 +/- 2.9 (11)
	5-HT3-A + 5-HT3-B	4.6 +/- 0.3 (33) ($p<5e^{-9}$)	7.3 +/- 0.3 (2)	15.8 +/- 1.6 (7) ($p<0.05$)	19.1 +/- 2.1 (10) ($p<0.005$)
Fast control 5-HT response	5-HT3-A	1.6 +/- 0.2 (11)	2.6 +/- 1.4 (2)	3.6 +/- 1.4 (7)	4.3 +/- 1.3 (5)
	5-HT3-A + 5-HT3-B	5.6 +/- 0.6 (12) ($p<5e^{-5}$)	9.7 +.- 1.6 (5) ($p<0.05$)	22.2 +/- 3.2 (11) ($p<1e^{-4}$)	17.5 +/- 1.6 (7) ($p<e^{-4}$)

The differences in kinetics and pharmacology observed in the Ca^{2+} influx experiments were not due to altered intracellular Ca^{2+} buffering in the Ca^{2+}-influx studies. Similar results were observed for 5-HT and 1-PBG-elicited whole-cell currents (Figure 9d). In cells expressing only 5-HT3-A receptors, 10 μM 1-PBG (solid bar) produced a small inward current and increase in conductance (Figure 9d top left). Cells were challenged with a voltage ramp protocol to simultaneously determine whole cell conductance changes (1 Hz); the ramp-induced currents (spikes in Figure 9d) are shown on a faster time scale in Figure 9e. The cell subsequently revealed a large 5-HT response (clear bar indicates when 10 μM 5-HT was applied). In double transfectants, the response to 10 μM 1-PBG was larger in cells that produced comparable 5-HT responses (Figure 9d top right). Top: inward currents elicited by 10 μM 1-PBG (solid bar) and 10 μM 5-HT (clear bar) from a holding potential of -68 mV. Bottom: inward currents elicited by 100 μM 1-PBG (hatched bar). 1-PBG (10 μM) elicited responses that were 3 +/- 1 % (n=7) of the response to 10 μM 5-HT in individual 5-HT3-A/HEK and 10-fold higher (28 and 34%) of the response to 10 μM 5-HT in individual 5-HT3-A/5-HT3-B/HEK cells. The rate of decay of the response to 5-HT (10 μM; Figure 9d top, clear bar) and 1-PBG (30 μM; Figure 9d bottom, hatched bar) was usually accelerated in the presence of 5-HT3-B.

The voltage dependence of the 5-HT induced current was rectifying in 5-HT3-A-expressing cells and more linear in the presence of 5-HT3-B (Figure 9e). 5-HT induced currents from transfected HEK293 cells expressing the homomeric receptor (left) and 5-HT3-A and 5-HT3-B subunits (right). A voltage ramp was applied to the cell from -120 to +80 mV (not corrected for a junction potential of -18 mV) over 200 msec (1 mV/ms) before (solid circle) and during (clear circle) agonist application. The V_{rev} were similar for both responses and were near 0 mV. Asterisks above the recordings in (d) indicate the ramp currents shown on an expanded scale in (e). The ratio of 5-HT induced current measured 50 mV positive and negative from V_{rev} was

calculated as an indication of the degree of rectification. In 5-HT3-A/HEK cells, the inward current was 2-fold larger than the outward current (ratio: 0.53 +/- 0.04; n=10). In cells expressing both subunits, the current-voltage relationship was linear (ratio: 1.07 +/- 0.10; n=5). This rectification difference observed between the two transfectants was statistically different ($p < 0.005$).

In all cell lines tested, the Ca^{+2} influx observed during challenge with agonists at concentrations near their EC50 was completely blocked by the specific 5-HT3 receptor antagonists tropisetron (IC50 ~ 10 nM), LY 278584 (IC50 ~6 nM) and MDL 72222 (IC50 ~ 15 nM). Ketanserin, an antagonist at 5-HT_2 receptors, had no effect on 5-HT or 1-PBG induced Ca^{2+} responses up to 10 μM. Spiperone, an antagonist at 5-HT_{2A} and D_2 DA receptors, appeared to be a partial agonist at the 5-HT3-A receptor at concentrations above 1 μM.

The mechanism underlying the altered kinetics in the presence of 5-HT3-B is not known. In one possibility, 5-HT3-B allosterically modulates the rate of desensitization. If this were the case, then one prediction is a decrease in the peak currents in 5-HT3-B containing receptors compared to homomeric receptors (which was not observed), but this prediction assumes no change in single channel conductance. Heteromeric channels in fact have a significantly larger single channel conductance compared to homomeric channels (Davies et al., 1999). In a second possibility, 5-HT3-B alters the rate for agonist binding such that the first latency is shorter in heteromers compared to homomers and the rate to desensitize is not altered. A similar model was presented for the sodium channel (Aldrich et al., 1983). The time between 80% peak values on the rising and falling phases of the response (t80) appeared to be proportional to the time to peak response, which is consistent with the latter mechanism. In the majority of oocytes, the time to peak was 2-fold faster if 5-HT3-A was co-injected with 5-HT3-B. Furthermore, the altered pharmacology is consistent with a decrease in first latency for channel opening. The effect observed (a decreased nH and a decreased EC_{50} in the presence of 5-HT3-B) may be explained by a loss of negative cooperativity (Liu and Dilger, 1993) when 5-HT3-A subunits

associate with 5-HT3-B subunits, which have no detectable functional binding site for agonists. In this model, binding of one agonist decreases the affinity of other agonist binding sites for agonist in the receptor complex. 5-HT3 receptors reveal a very strong cooperativity—Hill coefficients have been determined from functional studies to be near 2 or 3 (Brown et al., 1998; Sepulveda et al., 1991; Zhong et al., 1999). In a model in which the first latency decreases while the rate of desensitization remains unchanged, there is predicted to be an increased peak current due to channel opening within a shorter time window, which was observed. Single channel analysis may be prohibitive in this system since homomeric receptors have sub-pS single channel conductances (Davies et al., 1999). Only the heteromeric receptors have a large enough single channel conductance to reliably measure single channel openings (Davies et al., 1999).

EXAMPLE 7

Binding assay on human 5-HT3-B and 5-HT3-A co-transfected mammalian cells.

HEK293 cells stably expressing 5-HT3-A receptor with or without human 5-HT3-B can be used in ^{3}H-[mCPBG] binding assays. Equilibrium ligand binding assays can be performed using conventional procedures (Lummis and Baker, 1997; Lummis et al., 1993). Specific ^{3}H-[mCPBG] binding is observed in membrane preparations from 5-HT3 receptor and human 5-HT3-B transfected cells. Oocytes expressing 5-HT3-A and human 5-HT3-B can be used to measure the affinity of binding of other compounds and their ability to displace ^{3}H-[mCPBG] binding.

EXAMPLE 8

Cloning of the human 5-HT3-B cDNA into E. coli Expression Vectors

Recombinant human 5-HT3-B is produced in E. coli following the transfer of the expression cassette into E. coli expression vectors, including but not limited to, the pET series (Novagen). The pET vectors place human 5-HT3-B expression under control of the tightly regulated bacteriophage T7 promoter. Following

transfer of this construct into an E. coli host that contains a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of human 5-HT3-B is induced when an appropriate lac substrate (IPTG) is added to the culture. The levels of expressed human 5-HT3-B are determined by the assays described herein.

The cDNA encoding the entire open reading frame for human 5-HT3-B is inserted into the NdeI site of pET [16]11a. Constructs in the positive orientation are identified by sequence analysis and used to transform the expression host strain BL21. Transformants are then used to inoculate cultures for the production of human 5-HT3-B protein. Cultures may be grown in M9 or ZB media, whose formulation is known to those skilled in the art. After growth to an OD_{600} to approximately 1.5, expression of human 5-HT3-B is induced with 1 mM IPTG for 3 hours at 37°C.

EXAMPLE 9

Cloning of human 5-HT3-B cDNA into a Baculovirus Expression Vector for Expression in Insect Cells

Baculovirus vectors, which are derived from the genome of the AcNPV virus, are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL# 1711). Recombinant baculoviruses expressing human 5-HT3-B cDNA is produced by the following standard methods (InVitrogen Maxbac Manual): the human 5-HT3-B cDNA constructs are ligated into the polyhedrin gene in a variety of baculovirus transfer vectors, including the pAC360 and the BlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA [Kitts, P.A., Nuc. Acid. Res. 18: 5667 (1990)] into Sf9 cells. Recombinant pAC360 viruses are identified by the absence of inclusion bodies in infected cells and recombinant pBlueBac viruses are identified on the basis of ß-galactosidase expression (Summers, M. D.

and Smith, G. E., Texas Agriculture Exp. Station Bulletin No. 1555). Following plaque purification, human 5-HT3-B expression is measured by the assays described herein.

The cDNA encoding the entire open reading frame for human 5-HT3-B is inserted into the BamHI site of pBlueBacII. Constructs in the positive orientation are identified by sequence analysis and used to transfect Sf9 cells in the presence of linear AcNPV mild type DNA.

Authentic, active human 5-HT3-B is found in the cytoplasm of infected cells. Active human 5-HT3-B is extracted from infected cells by hypotonic or detergent lysis.

EXAMPLE 10

Cloning of human 5-HT3-B cDNA into a yeast expression vector

Recombinant human 5-HT3-B is produced in the yeast S. cerevisiae following the insertion of the optimal human 5-HT3-B cDNA cistron into expression vectors designed to direct the intracellular or extracellular expression of heterologous proteins. In the case of intracellular expression, vectors such as EmBLyex4 or the like are ligated to the human 5-HT3-B cistron [Rinas, U. et al., Biotechnology 8: 543-545 (1990); Horowitz B. et al., J. Biol. Chem. 265: 4189-4192 (1989)]. For extracellular expression, the human 5-HT3-B cistron is ligated into yeast expression vectors which fuse a secretion signal (a yeast or mammalian peptide) to the NH_2 terminus of the human 5-HT3-B protein [Jacobson, M. A., Gene 85: 511-516 (1989); Riett L. and Bellon N. Biochem. 28: 2941-2949 (1989)].

These vectors include, but are not limited to pAVE1>6, which fuses the human serum albumin signal to the expressed cDNA [Steep O. Biotechnology 8: 42-46 (1990)], and the vector pL8PL which fuses the human lysozyme signal to the expressed cDNA [Yamamoto, Y., Biochem. 28: 2728-2732)]. In addition,

human 5-HT3-B is expressed in yeast as a fusion protein conjugated to ubiquitin utilizing the vector pVEP [Ecker, D. J., J. Biol. Chem. 264: 7715-7719 (1989), Sabin, E. A., Biotechnology 7: 705-709 (1989), McDonnell D. P., Mol. Cell Biol. 9: 5517-5523 (1989)]. The levels of expressed human 5-HT3-B are determined by the assays described herein.

EXAMPLE 11

Purification of Recombinant human 5-HT3-B

Recombinantly produced human 5-HT3-B may be purified by antibody affinity chromatography.

Human 5-HT3-B antibody affinity columns are made by adding the anti-human 5-HT3-B antibodies to Affigel-10 (Bio-Rad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents and the cell culture supernatants or cell extracts containing solubilized human 5-HT3-B are slowly passed through the column. The column is then washed with phosphate- buffered saline together with detergents until the optical density (A280) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) together with detergents. The purified human 5-HT3-B protein is then dialyzed against phosphate buffered saline.

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WHAT IS CLAIMED IS:

1. An isolated and purified DNA molecule which encodes human 5-HT3-B protein, or a functional derivative thereof, wherein said protein functions as a human serotonin receptor subunit.

2. The isolated and purified DNA molecule of claim 1, having a nucleotide sequence selected from a group consisting of: **(SEQ.ID.NO.:1)**; **(SEQ.ID.NO.:2)**; and functional derivatives thereof.

3. The isolated and purified DNA molecule of claim 1, wherein said DNA molecule is genomic DNA.

4. An expression vector for expression of human 5-HT3-B protein in a recombinant host, wherein said vector contains a recombinant gene encoding human 5-HT3-B protein according to claim 1, or a functional derivative thereof.

5. The expression vector of claim 4, wherein the expression vector contains a cloned gene encoding human 5-HT3-B protein wherein said protein functions as a human serotonin receptor subunit, having a nucleotide sequence selected from a group consisting of: (SEQ.ID.NO.:1); (SEQ.ID.NO.:2); and functional derivatives thereof.

6. The expression vector of claim 4, wherein the expression vector contains genomic DNA encoding human 5-HT3-B protein.

7. A recombinant host cell containing a recombinantly cloned gene encoding human 5-HT3-B protein wherein said protein functions as a human serotonin receptor subunit, or functional derivative thereof.

8. The recombinant host cell of claim 7, wherein said gene has a nucleotide sequence selected from a group consisting of: (SEQ.ID.NO.:1); (SEQ.ID.NO.:2); and functional derivatives thereof.

9. The recombinant host cell of claim 7, wherein said cloned gene encoding human 5-HT3-B protein is genomic DNA.

10. A protein, in substantially pure form which functions as human 5-HT3-B protein and wherein said protein functions as a modifier of the human 5-HT3-A receptor.

11. The protein according to claim 10, having an amino acid sequence selected from a group consisting of: (SEQ.ID.NO.:3); (SEQ.ID.NO.:4); and functional derivatives thereof.

12. A monospecific antibody immunologically reactive with human 5-HT3-B protein wherein said protein functions as a modifier of the human 5-HT3-A receptor.

13. The antibody of Claim 12, wherein the antibody blocks activity of the 5-HT3-B subunit of the human serotonin receptor.

14. A process for expression of human 5-HT3-B protein in a recombinant host cell, comprising:

(a) transferring the expression vector of Claim 4 into suitable host cells; and

(b) culturing the host cells of step (a) under conditions which allow expression of the human 5-HT3-B protein from the expression vector.

15. A method of identifying compounds that modulate human 5-HT3-B protein activity, comprising:

(a) combining a modulator of human 5-HT3-B protein activity with human 5-HT3-B protein wherein said protein optionally functions as a modifier of the human 5-HT3-A receptor; and

(b) measuring an effect of the modulator on the protein.

16. The method of claim 15, wherein the effect of the modulator on the protein is inhibiting or enhancing binding of human 5-HT3 receptor ligands.

17. The method of claim 15, wherein the effect of the modulator on the protein is stimulation or inhibition of human 5-HT3-B-containing serotonin receptor.

18. The method of claim 17, wherein the human 5-HT3-B is altering the kinetics and the pharmacology of the human 5-HT3-B-containing serotonin receptor.

19. A compound active in the method of Claim 15, wherein said compound is a modulator of a human 5-HT3-B containing serotonin receptor.

20. A compound active in the method of Claim 15, wherein said compound is an agonist or antagonist of a subclass of 5-HT3 receptor consisting of both 5-HT3-A and 5HT3B proteins.

21. A compound active in the method of Claim 15, wherein said compound is a modulator of expression of a 5-HT3-B subunit.

22. A pharmaceutical composition comprising a compound active in the method of Claim 15, wherein said compound is a modulator of human 5-HT3-B subunit activity.

23. A method of treating a patient in need of such treatment for a condition which is mediated by a human 5-HT3-B-containing serotonin receptor , comprising administration of a human 5-HT3-B modulating compound active in the method of Claim 15.

5

FIG. 1

[SEQ.ID.NO.:5]
Nucleic Acid sequence of the human 5-HT3-B
(full sequence including untranslated regions;
1923 bases. (141 bases of 5' UTR; 456 bases of 3' UTR)

CCACGCGTCCGTAAGGATAGCATCAACTGGCAAACGGAGAAGGAGGAGAA
CAGAGTGGAGAGGAACCCTGTTAGGAGAAATTGAGCGGCATTCCATCTGG
TAGGCAAGTTTGCATTTCTCCTTTTTGGGATCTGCCCAGGA**ATG**TTGTCA
AGTGTAATGGCTCCCCTGTGGGCCTGCATCCTGGTGGCTGCAGGAATTCT
AGCCACAGATACACATCATCCCCAGGATTCTGCTCTGTATCATCTCAGCA
AGCAGCTATTACAGAAATATCATAAAGAAGTGAGACCTGTTTACAACTGG
ACCAAGGCCACCACAGTCTACCTGGACCTGTTCGTCCATGCTATATTGGA
TGTGGATGCAGAGAATCAAATATTAAAGACAAGTGTATGGTACCAAGAGG
TCTGGAATGATGAATTTTTATCCTGGAACTCCAGCATGTTTGATGAGATT
AGAGAGATCTCCCTACCTCTAAGTGCCATCTGGGCCCCCGATATCATCAT
CAATGAGTTTGTGGACATTGAAAGATACCCTGACCTTCCCTATGTTTATG
TGAACTCATCTGGGACCATTGAGAACTATAAGCCCATCCAGGTGGTCTCT
GCGTGCAGTTTAGAGACATATGCTTTTCCATTTGATGTCCAGAATTGCAG
CCTGACCTTCAAGAGCATTCTGCATACAGTGGAAGACGTAGACCTGGCCT
TTCTGAGGAGCCCAGAAGACATTCAGCATGACAAAAAGGCGTTTTTGAAT
GACAGTGAGTGGGAACTTCTATCTGTGTCCTCCACATACAGCATCCTGCA
GAGCAGCGCTGGAGGATTTGCACAGATTCAGTTTAATGTGGTGATGCGCA
GGCACCCCCTGGTCTATGTCGTGAGTCTGCTGATTCCTAGCATCTTTCTC
ATGCTGGTGGACCTGGGGAGCTTCTACCTGCCACCCAACTGCCGAGCCAG
GATTGTGTTCAAGACCAGTGTGCTGGTGGGCTACACCGTCTTCAGGGTCA
ACATGTCCAACCAGGTGCCACGGAGTGTAGGGAGCACCCCTCTGATTGGG
CACTTCTTCACCATCTGCATGGCCTTCTTGGTTCTCAGCTTAGCTAAGTC
CATCGTGTTGGTCAAATTCCTCCATGATGAGCAGCGTGGTGGACAGGAGC
AGCCCTTCTTGTGCCTTCGAGGGGACACCGATGCTGACAGGCCTAGAGTG
GAACCCAGGGCCCAACGTGCTGTGGTAACAGAGTCCTCGCTGTATGGAGA
GCACCTGGCCCAGCCAGGAACCCTGAAGGAAGTCTGGTCGCAGCTTCAAT
CTATCAGCAACTACCTCCAAACTCAGGACCAGACAGACCAACAGGAGGCA
GAGTGGCTGGTCCTCCTGTCCCGCTTTGACCGACTGCTCTTCCAAAGCTA
CCTTTTCATGCTGGGGATCTACACCATCACTCTGTGCTCCCTCTGGGCAC
TGTGGGGCGGCGTG**TGA**AGACTGAAGTGTTCTTCAGTAATTGTGCTGGCA
CTTAGGAGAGAGAGAGGGGGAATAATAGTGGGTTAAAAAGCTTTCTGGGT
CGGGTGTGGTGGTTCTTGCCTATAGTCCCAGTGCTTTGGGAGGCCATAGC
AGGAGGATTGCTTGAGCCCAGGAGTTCGAGACCAGCCAGAGCAACATAGT
GAGACCACATCTCTACCAGTAAATAAATAAATAAATAAATAAATAAATAA
ATAAATAAATAGCTGGGCATAGTGGCTCATGCCTGTACTCTCAGCTACTT
GGGAGGTTGAGGTGGGAGGATTGCTTGAGCCCAGGATTTCAAGGCTGCAG
TGAGCCATGATTGCACCACTGCACCCCAGCCTGGGTGACAGAACAAGACC
CTGTCTCAAAAAAAAAATAAAATAAAAGGCTTTCTGCCTTCAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAG

FIG. 2

[SEQ.ID.NO.:6]
Nucleotide sequence for the coding region of 5-HT3-B is shown; 1326 bases.

ATGTTGTCAAGTGTAATGGCTCCCCTGTGGGCCTGCATCCTGGTGGCTGC
AGGAATTCTAGCCACAGATACACATCATCCCCAGGATTCTGCTCTGTATC
ATCTCAGCAAGCAGCTATTACAGAAATATCATAAAGAAGTGAGACCTGTT
TACAACTGGACCAAGGCCACCACAGTCTACCTGGACCTGTTCGTCCATGC
TATATTGGATGTGGATGCAGAGAATCAAATATTAAAGACAAGTGTATGGT
ACCAAGAGGTCTGGAATGATGAATTTTTATCCTGGAACTCCAGCATGTTT
GATGAGATTAGAGAGATCTCCCTACCTCTAAGTGCCATCTGGGCCCCCGA
TATCATCATCAATGAGTTTGTGGACATTGAAAGATACCCTGACCTTCCCT
ATGTTTATGTGAACTCATCTGGGACCATTGAGAACTATAAGCCCATCCAG
GTGGTCTCTGCGTGCAGTTTAGAGACATATGCTTTTCCATTTGATGTCCA
GAATTGCAGCCTGACCTTCAAGAGCATTCTGCATACAGTGGAAGACGTAG
ACCTGGCCTTTCTGAGGAGCCCAGAAGACATTCAGCATGACAAAAAGGCG
TTTTTGAATGACAGTGAGTGGGAACTTCTATCTGTGTCCTCCACATACAG
CATCCTGCAGAGCAGCGCTGGAGGATTTGCACAGATTCAGTTTAATGTGG
TGATGCGCAGGCACCCCCTGGTCTATGTCGTGAGTCTGCTGATTCCTAGC
ATCTTTCTCATGCTGGTGGACCTGGGGAGCTTCTACCTGCCACCCAACTG
CCGAGCCAGGATTGTGTTCAAGACCAGTGTGCTGGTGGGCTACACCGTCT
TCAGGGTCAACATGTCCAACCAGGTGCCACGGAGTGTAGGGAGCACCCCT
CTGATTGGGCACTTCTTCACCATCTGCATGGCCTTCTTGGTTCTCAGCTT
AGCTAAGTCCATCGTGTTGGTCAAATTCCTCCATGATGAGCAGCGTGGTG
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CAGGAGGCAGAGTGGCTGGTCCTCCTGTCCCGCTTTGACCGACTGCTCTT
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TCTGGGCACTGTGGGGCGGCGTGTGA

FIG. 3

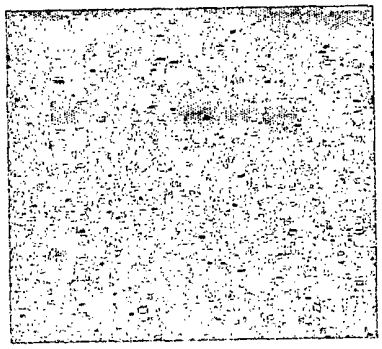
[SEQ.ID.NO.:7]
The amino acid sequence of 5-HT3-B is shown (441 amino acids).

Met Leu Ser Ser Val Met Ala Pro Leu Trp Ala Cys Ile Leu Val Ala Ala Gly Ile
Leu Ala Thr Asp Thr His His Pro Gln Asp Ser Ala Leu Tyr His Leu Ser
Lys Gln Leu Leu Gln Lys Tyr His Lys Glu Val Arg Pro Val Tyr Asn Trp Thr
Lys Ala Thr Thr Val Tyr Leu Asp Leu Phe Val His Ala Ile Leu Asp Val Asp
Ala Glu Asn Gln Ile Leu Lys Thr Ser Val Trp Tyr Gln Glu Val Trp Asn Asp
Glu Phe Leu Ser Trp Asn Ser Ser Met Phe Asp Glu Ile Arg Glu Ile Ser Leu
Pro Leu Ser Ala Ile Trp Ala Pro Asp Ile Ile Ile Asn Glu Phe Val Asp Ile
Glu Arg Tyr Pro Asp Leu Pro Tyr Val Tyr Val Asn Ser Ser Gly Thr Ile Glu
Asn Tyr Lys Pro Ile Gln Val Val Ser Ala Cys Ser Leu Glu Thr Tyr Ala Phe
Pro Phe Asp Val Gln Asn Cys Ser Leu Thr Phe Lys Ser Ile Leu His Thr Val
Glu Asp Val Asp Leu Ala Phe Leu Arg Ser Pro Glu Asp Ile Gln His Asp Lys
Lys Ala Phe Leu Asn Asp Ser Glu Trp Glu Leu Leu Ser Val Ser Ser Thr Tyr
Ser Ile Leu Gln Ser Ser Ala Gly Gly Phe Ala Gln Ile Gln Phe Asn Val Val
Met Arg Arg His Pro Leu Val Tyr Val Val Ser Leu Leu Ile Pro Ser Ile Phe
Leu Met Leu Val Asp Leu Gly Ser Phe Tyr Leu Pro Pro Asn Cys Arg Ala Arg
Ile Val Phe Lys Thr Ser Val Leu Val Gly Tyr Thr Val Phe Arg Val Asn Met
Ser Asn Gln Val Pro Arg Ser Val Gly Ser Thr Pro Leu Ile Gly His Phe Phe
Thr Ile Cys Met Ala Phe Leu Val Leu Ser Leu Ala Lys Ser Ile Val Leu Val
Lys Phe Leu His Asp Glu Gln Arg Gly Gly Gln Glu Gln Pro Phe Leu Cys Leu
Arg Gly Asp Thr Asp Ala Asp Arg Pro Arg Val Glu Pro Arg Ala Gln Arg Ala
Val Val Thr Glu Ser Ser Leu Tyr Gly Glu His Leu Ala Gln Pro Gly Thr Leu
Lys Glu Val Trp Ser Gln Leu Gln Ser Ile Ser Asn Tyr Leu Gln Thr Gln Asp
Gln Thr Asp Gln Gln Glu Ala Glu Trp Leu Val Leu Leu Ser Arg Phe Asp Arg
Leu Leu Phe Gln Ser Tyr Leu Phe Met Leu Gly Ile Tyr Thr Ile Thr Leu Cys
Ser Leu Trp Ala Leu Trp Gly Gly Val

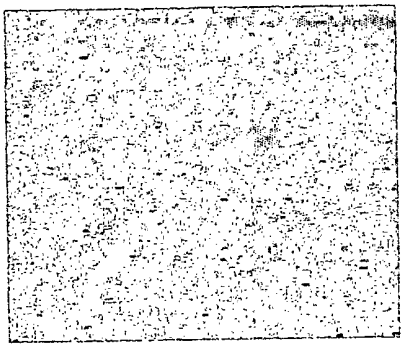
FIG. 4A

The tissue distribution of 5-HT3-B is shown.

1 2 3 4 5 6 7 8

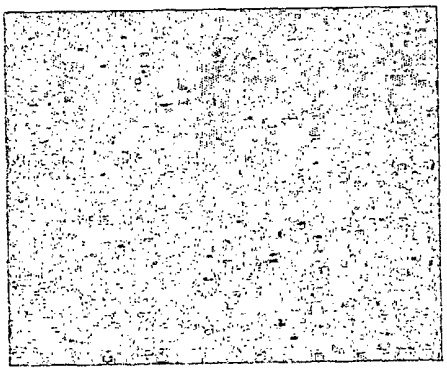


9 10 11 12 1 3 14 15



16 17 18 19 20 21 22 23

24 25 26 27 28 29 30 31



1, Cerebellum; 2, Cerebral cortex; 3, Medulla;
4, Spinal Cord; 5, Occipital pole; 6, frontal lobe;
7, Temporal lobe; 8, Putamen; 9, Amygdala;
10, Caudate nucleus; 11, Corpus callosum; 12, Hippocampus;
13, Whole brain; 14, Substantia nigra; 15, Thalamus;
16, Heart; 17, Brain; 18, Placenta; 19, Lung; 20, Liver;
21, Skeletal muscle; 22, Kidney; 23, Pancreas;
24, Spleen; 25, Thymus; 26, Prostate; 27, Testis;
28, Ovary; 29, Small Intestine; 30, Colon (mucosal lining);
31, Peripheral blood leukocyte.

FIG. 4B

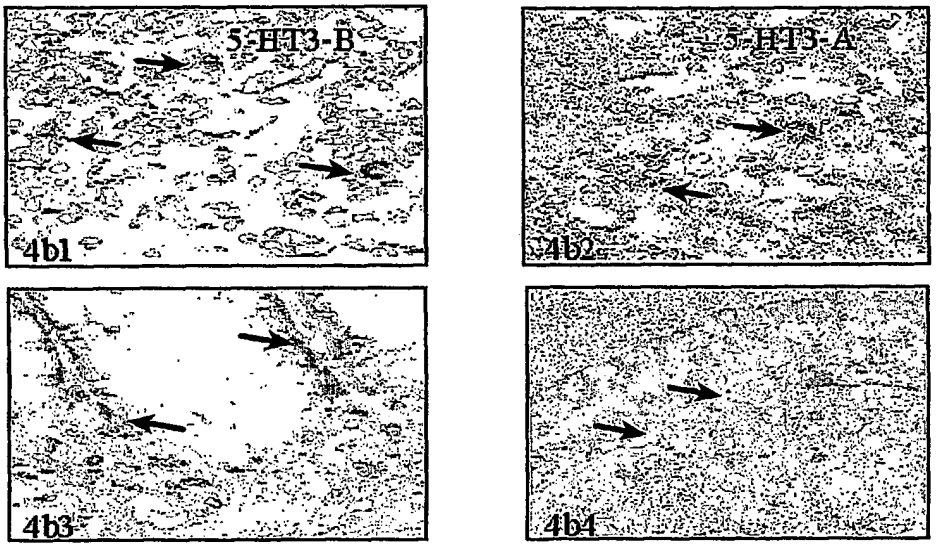


FIG. 4C

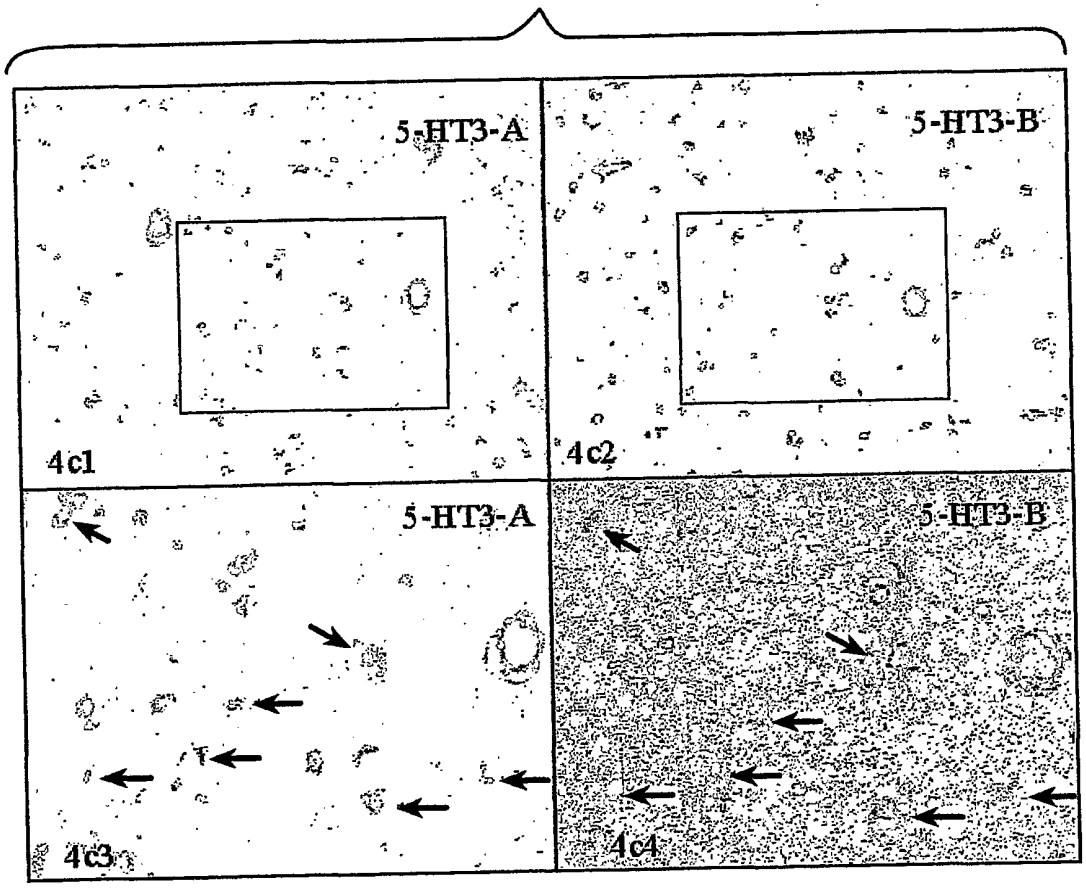


FIG. 5A

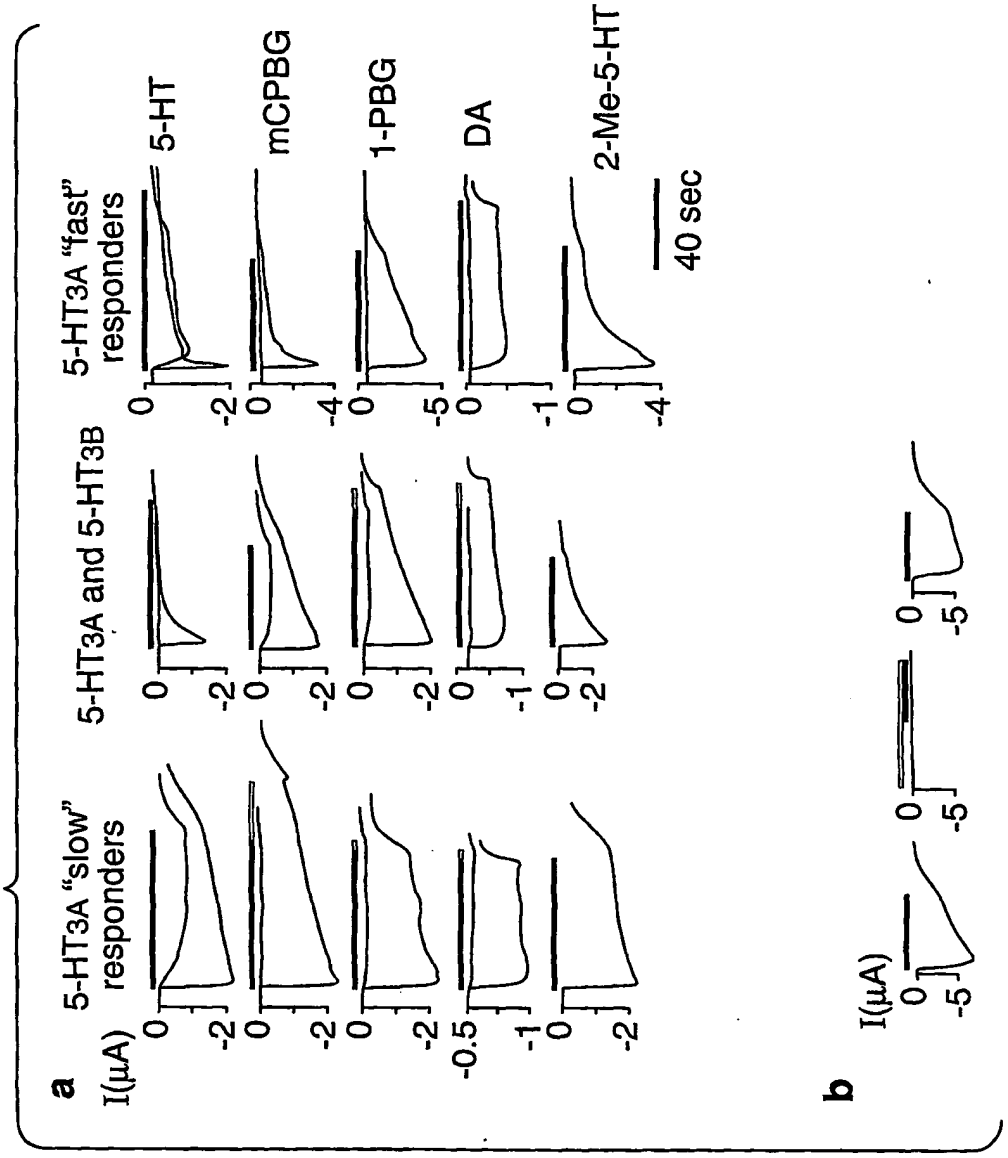

a
I(μA)
5-HT3A "slow" responders
5-HT3A and 5-HT3B
5-HT3A "fast" responders
5-HT
mCPBG
1-PBG
DA
2-Me-5-HT
40 sec
b
I(μA)

FIG. 5B

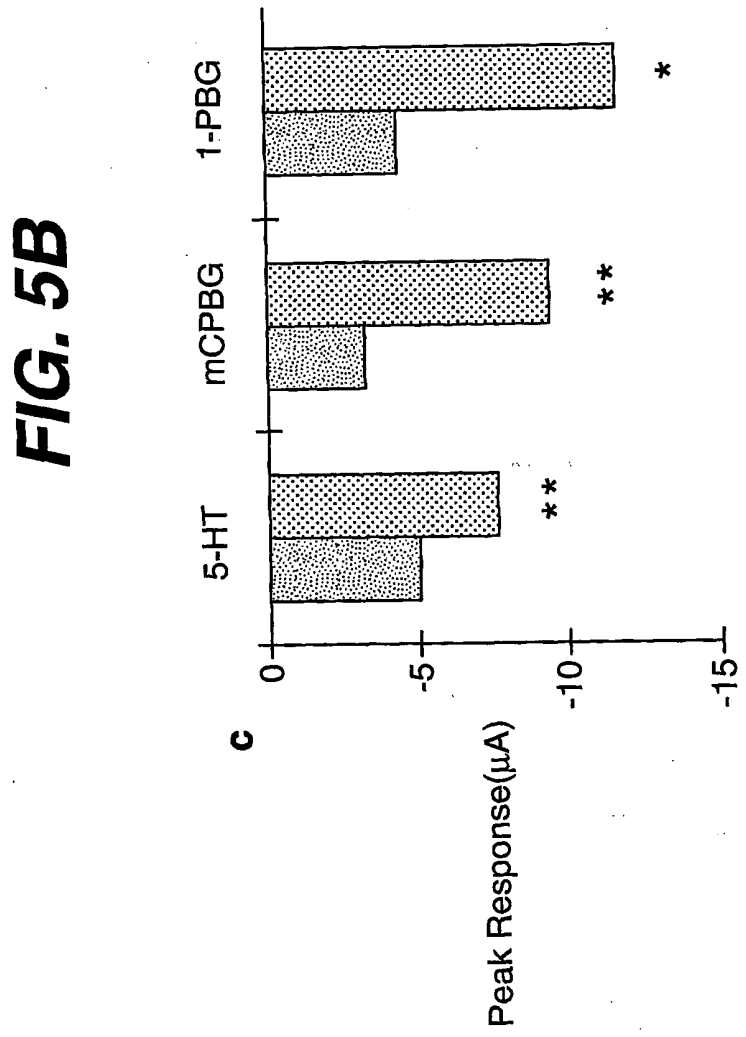


FIG. 6

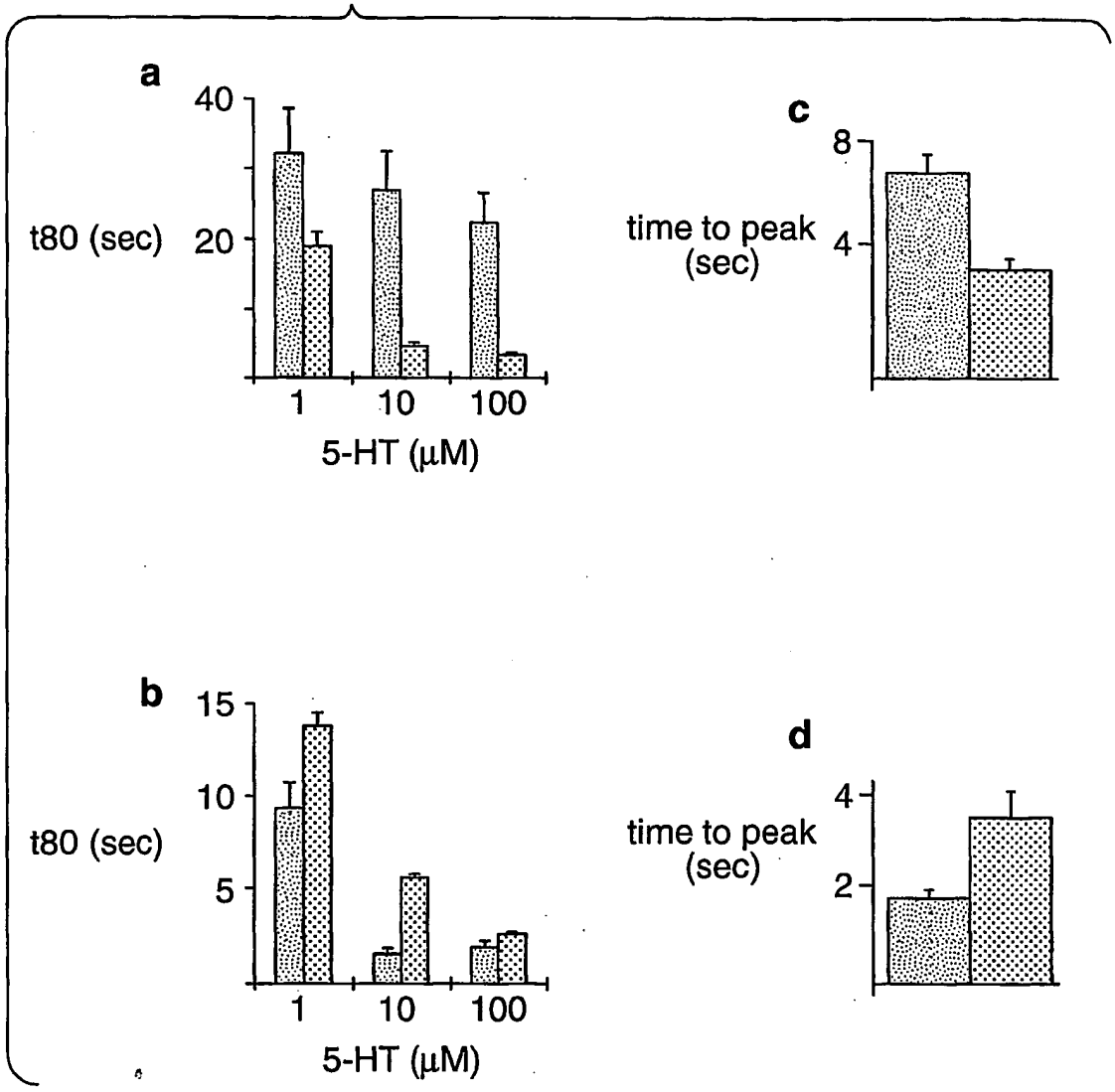

a
40
20
t80 (sec)
1
10
100
5-HT (μM)
c
8
4
time to peak (sec)
b
15
10
5
t80 (sec)
1
10
100
5-HT (μM)
d
4
2
time to peak (sec)

FIG. 7

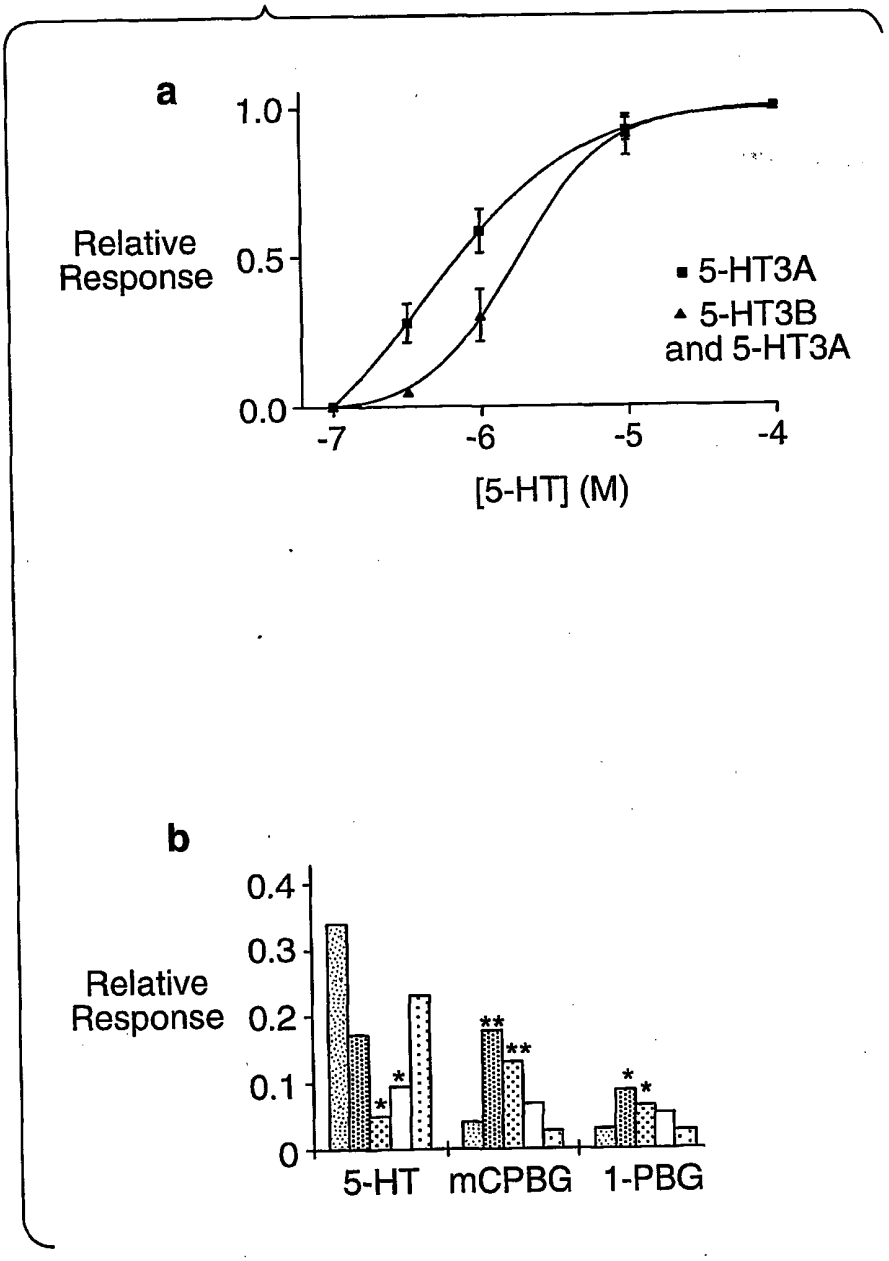

a
1.0
0.5
0.0
Relative Response
-7
-6
-5
-4
[5-HT] (M)
5-HT3A
5-HT3B and 5-HT3A
b
0.4
0.3
0.2
0.1
0
Relative Response
5-HT
mCPBG
1-PBG

FIG. 8

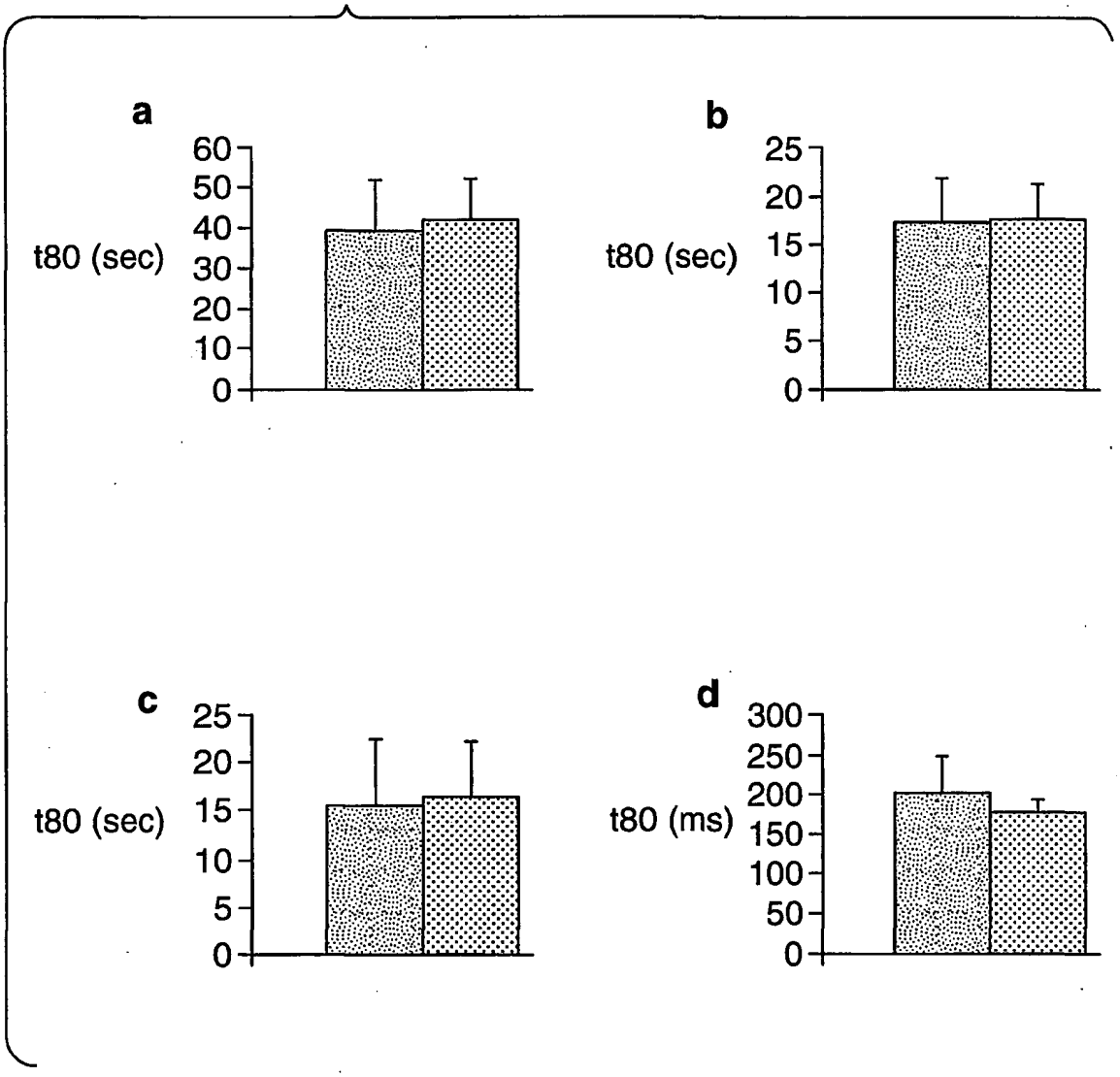

a
t80 (sec)
60
50
40
30
20
10
0
b
t80 (sec)
25
20
15
10
5
0
c
t80 (sec)
25
20
15
10
5
0
d
t80 (ms)
300
250
200
150
100
50
0

FIG. 9A

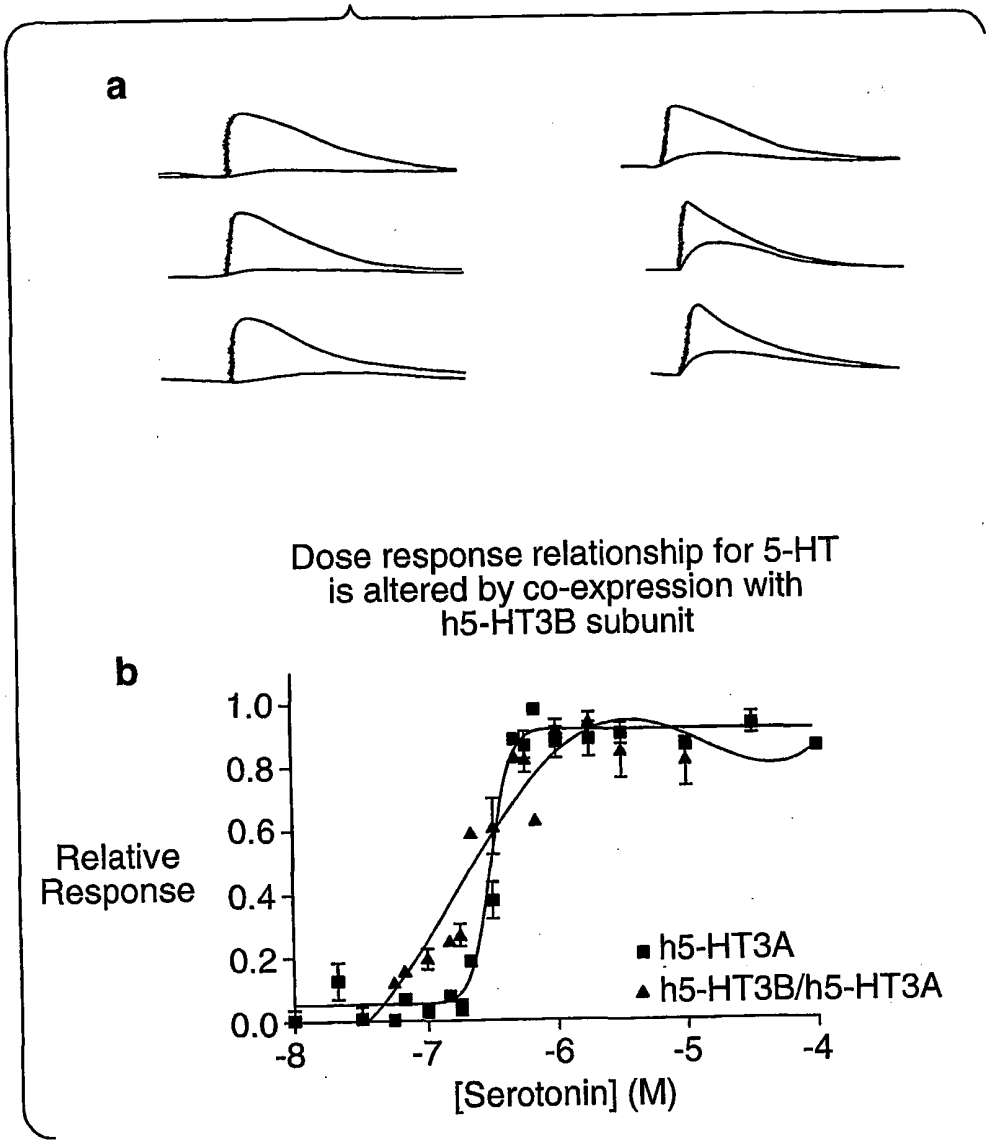
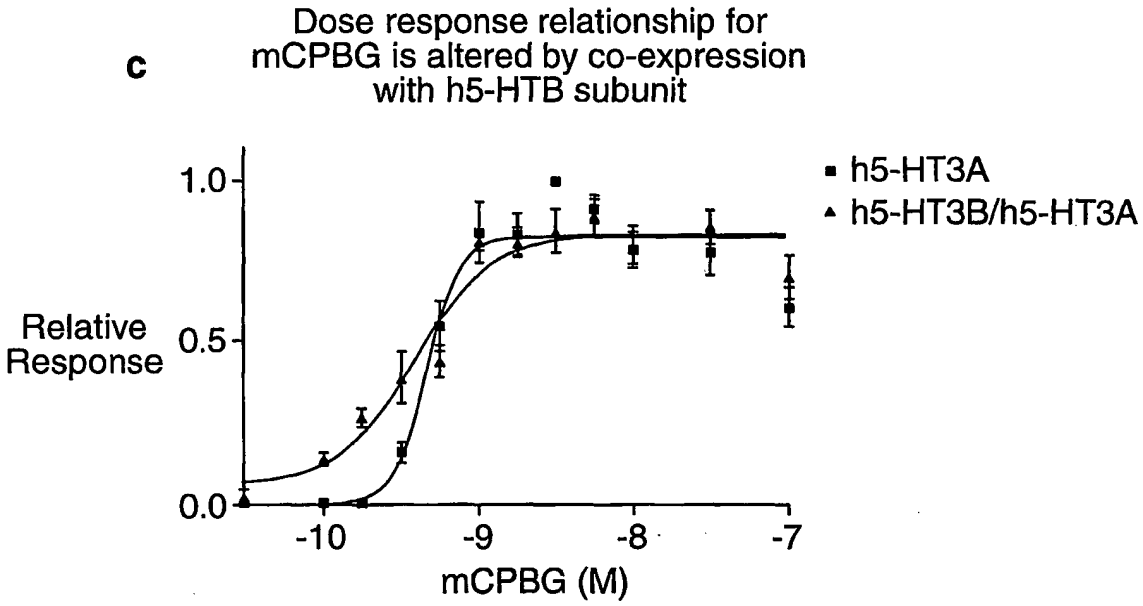

a
Dose response relationship for 5-HT
is altered by co-expression with
h5-HT3B subunit
b
Relative
Response
1.0
0.8
0.6
0.4
0.2
0.0
-8
-7
-6
-5
-4
[Serotonin] (M)
h5-HT3A
h5-HT3B/h5-HT3A

FIG. 9B


c
Dose response relationship for
mCPBG is altered by co-expression
with h5-HTB subunit
Relative
Response
1.0
0.5
0.0
-10
-9
-8
-7
mCPBG (M)
h5-HT3A
h5-HT3B/h5-HT3A

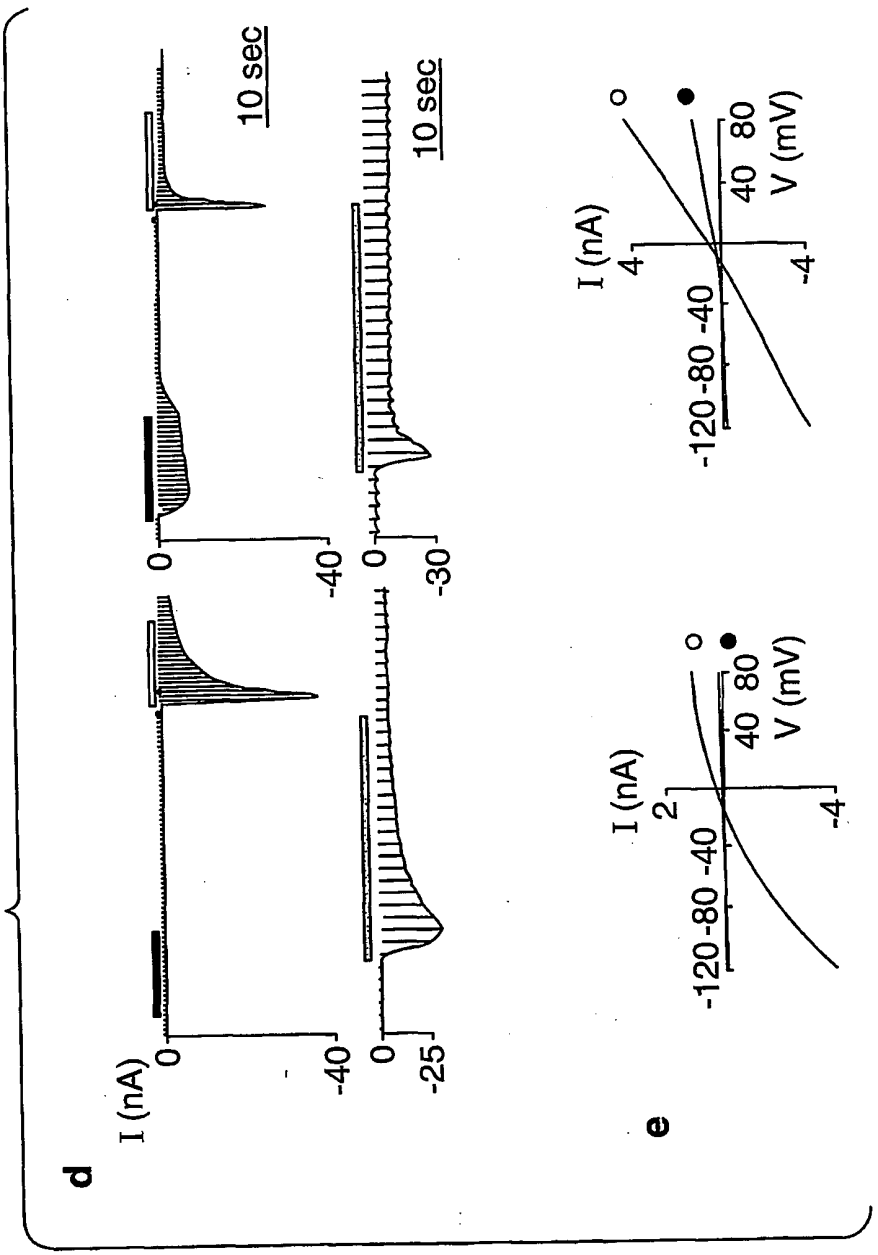

d
I (nA)
0
-40
0
-25
0
10 sec
-40
0
10 sec
-30
e
I (nA)
2
-120 -80 -40
40 80
V (mV)
-4
I (nA)
4
-120 -80 -40
40 80
V (mV)
-4

FIG. 9C

ORT1004

SEQUENCE LISTING

<110> Dubin, Adrienne
D'Andrea, Mike
Pyati, Jaushree
Zhu, Jessica Y
Erlander, Mark G

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<130> ORT-1004

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<170> PatentIn Ver. 2.0

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ORT1004

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ctggtggctg caggaattct agccacagat acacatcatc cccaggattc tgctctgtat 240
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accaaggcca ccacagtcta cctggacctg ttcgtccatg ctatattgga tgtggatgca 360
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tcctggaact ccagcatgtt tgatgagatt agagagatct ccctacctct aagtgccatc 480
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aag 1923

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<211> 441
<212> PRT
<213> Homo sapiens

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1 5 10 15

Ala Gly Ile Leu Ala Thr Asp Thr His His Pro Gln Asp Ser Ala Leu
20 25 30

Tyr His Leu Ser Lys Gln Leu Leu Gln Lys Tyr His Lys Glu Val Arg
35 40 45

Pro Val Tyr Asn Trp Thr Lys Ala Thr Thr Val Tyr Leu Asp Leu Phe

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     50                  55                  60

Val His Ala Ile Leu Asp Val Asp Ala Glu Asn Gln Ile Leu Lys Thr
 65                  70                  75                  80

Ser Val Trp Tyr Gln Glu Val Trp Asn Asp Glu Phe Leu Ser Trp Asn
                 85                  90                  95

Ser Ser Met Phe Asp Glu Ile Arg Glu Ile Ser Leu Pro Leu Ser Ala
            100                 105                 110

Ile Trp Ala Pro Asp Ile Ile Ile Asn Glu Phe Val Asp Ile Glu Arg
        115                 120                 125

Tyr Pro Asp Leu Pro Tyr Val Tyr Val Asn Ser Ser Gly Thr Ile Glu
    130                 135                 140

Asn Tyr Lys Pro Ile Gln Val Val Ser Ala Cys Ser Leu Glu Thr Tyr
145                 150                 155                 160

Ala Phe Pro Phe Asp Val Gln Asn Cys Ser Leu Thr Phe Lys Ser Ile
                165                 170                 175

Leu His Thr Val Glu Asp Val Asp Leu Ala Phe Leu Arg Ser Pro Glu
            180                 185                 190

Asp Ile Gln His Asp Lys Lys Ala Phe Leu Asn Asp Ser Glu Trp Glu
        195                 200                 205

Leu Leu Ser Val Ser Ser Thr Tyr Ser Ile Leu Gln Ser Ser Ala Gly
    210                 215                 220

Gly Phe Ala Gln Ile Gln Phe Asn Val Val Met Arg Arg His Pro Leu
225                 230                 235                 240

Val Tyr Val Val Ser Leu Leu Ile Pro Ser Ile Phe Leu Met Leu Val
                245                 250                 255

Asp Leu Gly Ser Phe Tyr Leu Pro Pro Asn Cys Arg Ala Arg Ile Val
            260                 265                 270

Phe Lys Thr Ser Val Leu Val Gly Tyr Thr Val Phe Arg Val Asn Met
        275                 280                 285

Ser Asn Gln Val Pro Arg Ser Val Gly Ser Thr Pro Leu Ile Gly His
    290                 295                 300

Phe Phe Thr Ile Cys Met Ala Phe Leu Val Leu Ser Leu Ala Lys Ser
305                 310                 315                 320

Ile Val Leu Val Lys Phe Leu His Asp Glu Gln Arg Gly Gly Gln Glu
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                325                 330                 335

Gln Pro Phe Leu Cys Leu Arg Gly Asp Thr Asp Ala Asp Arg Pro Arg
            340                 345                 350

Val Glu Pro Arg Ala Gln Arg Ala Val Val Thr Glu Ser Ser Leu Tyr
        355                 360                 365

Gly Glu His Leu Ala Gln Pro Gly Thr Leu Lys Glu Val Trp Ser Gln
    370                 375                 380

Leu Gln Ser Ile Ser Asn Tyr Leu Gln Thr Gln Asp Gln Thr Asp Gln
385                 390                 395                 400

Gln Glu Ala Glu Trp Leu Val Leu Leu Ser Arg Phe Asp Arg Leu Leu
                405                 410                 415

Phe Gln Ser Tyr Leu Phe Met Leu Gly Ile Tyr Thr Ile Thr Leu Cys
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Ser Leu Trp Ala Leu Trp Gly Gly Val
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<210> 9
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oligonucleotide

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<210> 10
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<212> DNA

ORT1004

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<220>
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oligonucleotide

<400> 10
tgttggtcaa attcctccat gatgagcagc gtggtggaca 40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/13071

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 1/00; C07H 21/04; C12N 1/20; C12P 21/06

US CL :530/350; 536/23.5; 435/252.3, 69.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.5; 435/252.3, 69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	DAVIES, P.A. et al., The 5-HT3b subunit is a major determinant of serotonin-receptor function. Leters to Nature. 28 January 1999, Vol. 397, pages 359-363, especially page 360 and attached sequence alignment.	1-11, 14-23 --- 12, 13
Y	US 5,766,879 A (GERALD et al.) 16 June 1998, especially col. 13.	12, 13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 DECEMBER 2000	Date of mailing of the international search report 03 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Michael Brannock Telephone No. (703) 308-0196

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PARALEGAL SPECIALIST
TECHNOLOGY CENTER 1600

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/13071

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

COMMERCIAL NUCLEIC ACID AND PROTEIN DATABASES
STN: MEDLINE, BIOSIS, BIOTECHNO
WEST 2.0
Search Terms: setotonin, 5-HT, receptor, clone, genomic

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-11 and 14, drawn to polynucleotides, polypeptides, vectors, host cells and methods of producing a polypeptide.
Group II, claim(s) 12-13, drawn to antibodies.
Group III, claim(s) 15-18, drawn to methods of identifying modulators of a polypeptide.
Group IV, claim(s)19-22, drawn to agonists and antagonists of a polypeptide.
Group V, claim(s) 23, drawn to a method of treatment.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under *PCT Rule 13.2, they lack the same or corresponding special technical features* for the following reasons: Pursuant to 37 C.F.R. 1.475(d), this Authority considers that the main invention in the instant application comprises the first recited product, a polynucleotide of SEQ ID NO: 1 and 2, and the first recited method of using that product, namely in the process of producing the encoded polypeptide of SEQ ID NO: 3 and. However, the examiner has noted that the SEQ ID NOs recited in the claims match the figure numbers and not those of the SEQ ID NOs provided in the sequence listing. It is assumed that the claims were intended to be directed to polynucleotides of SEQ ID NO: 4 and 5 encoding a polypeptide of SEQ ID NO: 7. Therefore, this authority considers the that the main invention in the instant application comprises the first recited product, a polynucleotide of SEQ ID NO: 4 and 5, and the first recited method of using that product, namely in the process of producing the encoded polypeptide of SEQ ID NO: 7. Note that there is no method of making the polynucleotide. Also included in this group is the product made, namely the encoded polypeptide of SEQ ID NO: 7, and vectors and host cells comprising the polynucleotide and expressed polypeptide. Further, pursuant to 37 C.F.R. 1.475(b)-(d), the ISA/US considers that the materially and functionally dissimilar products of Groups II and IV and the additional methods of Groups III and V do not correspond to the main invention. This Authority therefore considers that the several inventions do not share a special technical feature within the meaning of PCT Rule 13.2 and thus do not relate to a single general inventive concept within the meaning of PCT Rule 13.1.